Characterisation of glutamate-gated chloride channels from Dirofilaria immitis

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CHARACTERISATION OF GLUTAMATE-GATED CHLORIDE CHANNELS FROM *DIROFILARIA IMMITIS*

Submitted by

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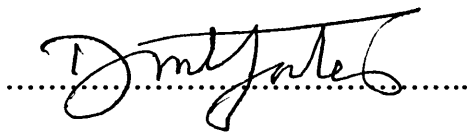

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Summary

Dirofilaria immitis is a parasitic nematode that infects dogs and causes cardiopulmonary disease. Members from both the closely related avermectin and milbemycin classes (the AMs) of anthelmintics are used in the preventative treatment of *D. immitis* infection. These drugs are able to kill larval stages of the nematode but are ineffective at killing the adult worms. Both classes appear to target the glutamate-gated chloride channels (GluCls), which are neurotransmitter receptors closely related to the $GABA_A$- and glycine-gated chloride channels. The objective of this project was to isolate and characterise GluCls from *D. immitis*, in an attempt to address why the AMs exhibit such contrasting potencies towards the larval and adult stages of the parasite.

Two cDNAs have been cloned that encode candidate *D. immitis* GluCl subunits. Based on homology searches, the subunits appear to be orthologues of the alternatively spliced GluClα3A and α3B subunits (from the *avr-14* gene) identified in both the free-living nematode *Caenorhabditis elegans* and the sheep parasite *Haemonchus contortus*. The pattern of alternative splicing appears conserved across the species examined, with the subunits possessing identical glutamate binding N-terminal domains, but differing channel-forming C-terminals. However, the processing of the mature GluClα3A mRNA appears to differ in *D. immitis* compared to *C. elegans* and *H. contortus*.

The *D. immitis* GluClα3 subunits failed to express at detectable levels in mammalian cell-lines. Therefore to investigate their pharmacology two-electrode voltage clamp recordings were made from *Xenopus* oocytes injected with subunit-specific cRNA. The GluClα3B subunits formed channels that were gated, in typical fashions, by glutamate (1-100mM) and IVM (1μM). Oocytes injected with GluClα3A cRNA failed to respond to glutamate; the response to IVM in these oocytes was not tested. The qualitative responses obtained were consistent with the pharmacology observed for the GluClα3 subunits in *C. elegans* and *H. contortus*. In addition, a glutamate dose response curve was generated for *C. elegans* GluClα3B homomeric channels expressed in oocytes. From the curve an EC_{50} of 2.2 ± 0.12mM and a Hill co-efficient of 0.72 ± 0.08 were determined. Whilst comparable to other

selected GluCls expressed in oocytes, these values suggest the CeGluClα3B subunits do not form homomers *in vivo*.

A novel LGIC subunit of unknown function, termed DG1, has also been cloned from *D. immitis*. The subunit exhibits low amino acid identity (25-30%) to GluCl and selected other LGIC subunits, but 50% identity to a predicted LGIC subunit (T27A1.4) from *C. elegans*. Initial RNAi experiments failed to establish a potential function for the T27A1.4 subunit.

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Abbreviations

ACh	acetylcholine
AChR	ACh receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMs	avermectins/milbemycins
AMV	alfalfa mosaic virus
ATP	adenosine 5′-triphosphate
dATP	deoxyadenosine 5′-triphosphate
AVM	avermectin
BIDN	3,3-bis-trifluoromethyl-bicyclo[2,2,1]heptane-2,2-dicarbonitrile
bp	base pairs
CKII	casein kinase II
dCTP	deoxycytosine 5′-triphosphate
DEC	diethylcarbamazine
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Eagles Media
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
cDNA	complimentary DNA
DTT	dithiothreitol
EC	effective concentration
EDTA	diaminoethanetetra-acetic acid
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GLR	non-NMDA-type glutamate receptor
GluCl	glutamate-gated chloride channel
dGTP	deoxyguanosine 5′-triphosphate
HBS	HEPES buffered saline
HEK	Human embryonic kidney
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
HisCl	histamine-gated chloride channels
ddH_2O	distilled deionised water
5-HT	5-hydroxytryptamine
IC	inhibitory concentration

IPTG	isopropyl-β-D-thiogalactopyranoside
IVM	ivermectin
$IVMPO_4$	ivermectin phosphate
kb	kilobases
K_D	dissociation constant
L_x	larval stage x
LB	Luria (Bertani) Broth
LBD	ligand binding domain
LGIC	ligand-gated ion channel
LGCC	ligand-gated chloride channel
MOPS	3-[N-Morpholino]propanesulfonic acid
NMDA	N-methyl D-aspartate
NMR	NMDA-type receptor
NMJ	neuromuscular junction
dNTPs	deoxynucleoside 5′-phosphates
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PKC	protein kinase C
PTX	picrotoxin
RACE	Rapid amplification of cDNA ends
RNA	ribonucleic acid
RNAi	RNA interference
cRNA	capped RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
rpm	revolutions per minute
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate (or lauryl sulphate)
SL1	spliced leader 1
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TEVC	two-electrode voltage clamp
TM	transmembrane region
dTTP	deoxythymidine 5′-triphosphate
UTR	untranslated region
UV	ultraviolet

Contents

Chapter 1

General Introduction

1.1 Nematodes

Nematodes, commonly termed "round worms", are the most populous metazoan animals on the planet, estimated by some to account for 80% of the total number (Platt, 1994). They are distinguished from other helminths (i.e. from flukes and tapeworms) by their non-segmented, cylindrical morphology, which along with their anatomy is highly conserved across species (White, 1988). Despite their body plan uniformity, which has bestowed nematodes with phylum status, i.e. the Nematoda, these worms exhibit considerable biological diversity (Blaxter *et al.*, 1998a). Approximately half the 20,000 described species of nematode, the large majority in terms of numerical abundance, are free-living; these worms typically only reach 1-2mm in length when adult, and can be found in both terrestrial (i.e. soil) and marine (both fresh and sea water) environments (Wood, 1988). A prime example of a free-living species is *Caenorhabditis elegans*, the "model" nematode (Blaxter, 1998b). As one of the species used in this study *C. elegans* is considered in more detail in section 1.4. The remaining species of nematode are parasitic, infecting a broad range of host organisms spanning both plant and animal (including vertebrates and invertebrates) kingdoms (Blaxter & Bird, 1997). Unlike their free-living counterparts certain parasitic species can reach considerable sizes, as illustrated below.

1.2 Parasitic nematodes

Nematodes are important parasites of humans, their domesticated animals and their crops. Of those nematodes that infect humans, a taxonomically diverse group of parasitic species termed the geohelminths are without doubt responsible for causing the most disease. Primary members of this group are *Ascaris lumbricoides*, *Trichuris trichiuria* and hookworm species. All of these nematodes have direct lifecycles, which means no intermediate hosts or vectors are required for their completion, but rather transmission occurs by faecal contamination of soil, foodstuffs and water supplies (Holland & Kennedy, 2002). Adult geohelminths reside within the intestine where they compete with the host for nutrients. Infection can lead to problems with appetite, growth, physical fitness, physical activity, work capacity, cognitive development and school performance in malnourished populations. In severe cases infection may also cause death (Kvalsvig, 2002; Stephenson, 2002). The singularly most important geohelminth is *A. lumbricoides* (the large gut roundworm), which has been recently estimated to infect over a quarter of the world's

population (1,472 million) (Crompton, 1999). Adult *A. lumbricoides* can reach up to 35cm, illustrating the considerable difference in size between free-living nematodes and certain parasitic species.

A second group of parasitic nematodes that have profound importance to human and also veterinary medicine are the filaria, which unlike the geohelminths, are taxonomically closely related (Blaxter *et al.*, 1998a; Dorris *et al.*, 1999). These nematodes are long-lived, tissue dwelling worms, which require biting arthropods to complete their lifecycles (Kazura, 2002); this is in contrast to the direct lifecycles of geohelminths. There are two major diseases attributed to filarial worms, lymphatic filariasis and onchocerciasis. Although not generally life threatening both conditions have serious socio-economic consequences. Lymphatic filariasis is principally caused by *Wuchereria bancrofti* (and to a lesser degree *Brugia* species) and affects 120 million people worldwide, mainly in sub-Saharan Africa and Asia (Crompton, 1999). Characteristically, affected individuals exhibit severe swelling of various body parts (lymphoedema), rendering them practically disabled (Kazura, 2002). Onchocerciasis (commonly termed river blindness) is the most severe result of infection with *Onchocerca volvulus.* Of the 18 million people infected with this parasite, mainly in sub-Saharan Africa (Crompton, 1999), 750,000 are either blind or severely disabled due to onchocercal eye diseases (Fischer & Büttner, 2002). Filarial nematodes are also important in veterinary medicine. Indeed, the species of major interest to this study is the filarial nematode *Dirofilaria immitis*, an important parasite of dogs (see section 1.3).

Nematodes are also important parasites of livestock, a chief example being *Haemonchus contortus*, which is a blood-sucking worm that infects sheep and other ruminants (Blaxter & Bird, 1997). *H. contortus* is a member of the group Strongylida that includes other parasites of veterinary importance such as *Trichostrongylus colubriformis*, a parasite of goats and sheep, and *Ostertagia ostertagi*, a parasite of cattle (Blaxter *et al.*, 1998a). Members of this family have direct lifecycles and reside within the intestine of their hosts. Infection causes anaemia, which leads to a reduction in the animal's condition and in certain cases death. The knock-on effect is reduced productivity and hence economic loss (Blaxter & Bird, 1997). There are increasing cases of drug resistance in these parasites, which is of growing concern in the livestock industry (Gill & Lacey, 1998; Kohler, 2001).

Plant parasitic nematodes cause billions of dollars worth of crop destruction per year. Although an old figure now, it has been estimated that they reduce the yield of the world's 40 major cash crops by approximately 12%. In poorer countries this can have a severe effect on malnutrition and the economics of the region. The main offenders responsible for the damage are the *Meloidogyne* species or "root-knot" nematodes, which are obligate root endo-parasites. After entering the roots they migrate to the developing vascular cylinder where they set-up a permanent feeding site (Blaxter & Bird, 1997).

1.3 *Dirofilaria immitis* ("Heartworm")

Dirofilaria immitis is a filarial nematode that primarily infects domesticated dogs and to a lesser degree domesticated cats; wild canines and ferrets can also act as potential reservoirs of infection. The adult nematodes usually reside within the infected animal's pulmonary arteries. However, they may also be found during chronic cases in the right side of the heart thus giving rise to their common name, "heartworm". There are a number of clinical manifestations associated with heartworm infection, the most significant being pulmonary hypertension, which in severe cases leads to right-sided heart failure (Knight, 1987). However, cases of heartworm are generally preventable and often curable with the application of the appropriate anthelmintic drugs (see section 1.7), although there are some limitations and problems associated with current treatments (Knight, 1998). Commercial interest in *D. immitis* and other such parasites is growing as the market for companion animal drugs expands (Witty, 1999; Meinke, 2001). The worldwide distribution of *D. immitis* across both temperate and tropical climates makes this parasite a prime target for the development of novel anthelmintics (Lok, 1988). Successfully improving existing treatments and developing new ones to combat *D. immitis* infections may also benefit how we deal with the closely related filarial nematodes that cause so much disease in humans, i.e. *W. bancrofti, O. volvulus* and *Brugia* species.

As stated, *D. immitis* is a filarial nematode and as such the ovoviviparous females, which reside in the pulmonary arteries, release (unsheathed) microfilariae (a pre-larval developmental stage) following successful fertilisation. Microfilariae are only ~300μm in length and circulate in the host's bloodstream until ingested by specific species of

mosquito. Within the mosquito microfilariae develop (through intermediate larval stages) to become infective third stage larvae (L_3), which are ~1.3mm in length. This period of development takes approximately 2-3 weeks. During a second blood meal the L_3s enter the primary host, moulting to become L_4s at the infection site before migrating towards the pulmonary arteries. En route the larvae undergo one further moult to become immature adults, sometimes referred to as L_5s. These worms reach the pulmonary arteries between 70-120 days post-infection. The immature adults at this point are 2-4cm in length and require several months of further development before attaining sexual maturity. This is reached when the adult worms reach 75% of their full-length; adult males reach 15-19cm whereas adult females reach 23-31cm. Microfilariae are observed circulating in the host's blood system at approximately 6.5 months post infection. *D. immitis* are long-lived parasites, having a mean life expectancy of 5-7 years (Knight, 1987). Their lifecycle is summarised in figure 1.1.

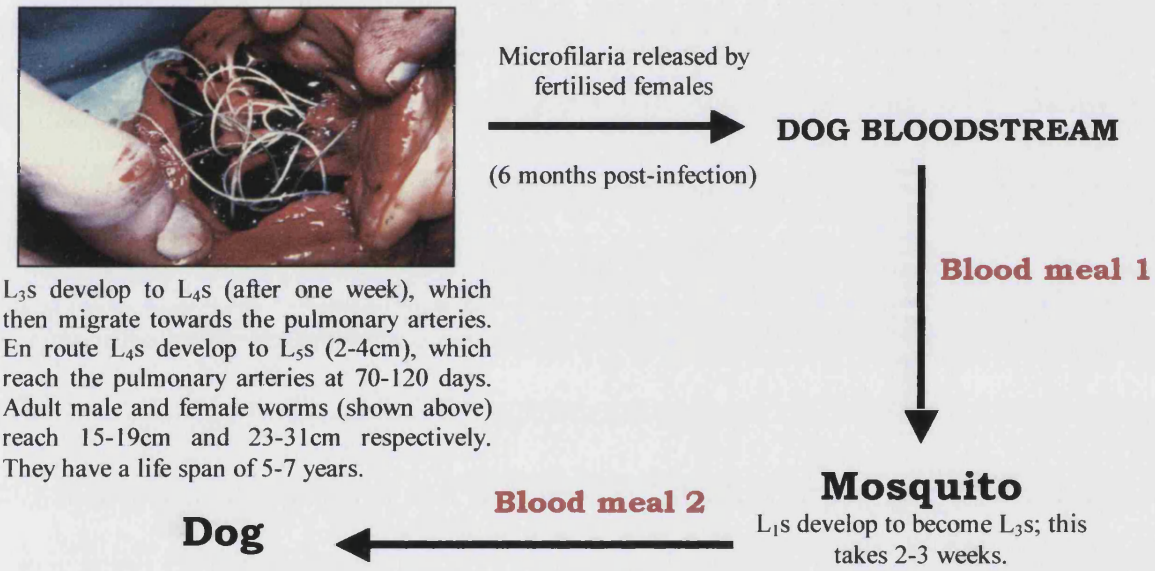


Figure 1.1. Lifecycle of *Dirofilaria immitis*. The image of the adult worms can be found at http://ucdnema.ucdavis.edu/imagemap/nemmap/ent156.html/slides/fromCD/1939/56B.Gif.

1.4 *Caenorhabditis elegans*

Caenorhabditis elegans is a small free-living species of nematode commonly found in soil, which feeds on anything it can fit into its mouth (usually bacteria) (Riddle *et al.*, 1997). In the 1960's Sydney Brenner selected *C. elegans* to address issues of animal development, particularly focusing on the nervous system (Brenner, 1973; Brenner, 1974). It has since

risen to become one of the most prominent model organisms in biological research. Indeed, it was the first metazoan to have its genome fully sequenced (The *C. elegans* Sequencing Consortium, 1998).

The two sexes, hermaphrodites and males, grow to 1-1.5mm in length and are only easily distinguished between when adult. Hermaphrodites, the more prevalent gender, reproduce by self-fertilisation and lay up to 300 eggs during their lifespan. As in other species of nematode, the hatched larvae undergo four stages of development to reach adulthood. This lifecycle takes only three days although once adult the worms may live for a further 2-3 weeks. Males arise spontaneously in the population at low frequency (~1 in 1000), but can also mate with hermaphrodites; hermaphrodites cannot mate with each other (Wood, 1988).

The inbreeding displayed by the self-fertilising hermaphrodites was perhaps the most important factor in the original decision by Brenner to use *C. elegans* in genetic studies, as it enables mutants to be easily maintained in the lab. The rare event of fertilisation by males can then be used to move mutations between strains. However, there are an increasing number of other reasons why *C. elegans* is appealing as a model organism. Their small size coupled to their rapid lifecycle facilitates cultivation and manipulation; the worms can be grown at 20°C on agar plates or in liquid cultures using *Escherichia coli* as their food source. They are anatomically simple (hermaphrodites have 959 somatic nuclei), with constancy of cell number and cell position; the complete wild-type cell lineage from fertilised egg to adult worm has been described (Riddle *et al.*, 1997). *C. elegans* are also genetically simple and as stated, their genome has been fully sequenced (The *C. elegans* Sequencing Consortium, 1998). This makes them amenable to a number of genetic screening processes (Jorgensen & Mango, 2002) and the increasingly popular technique of RNA interference (RNAi) (Hannon, 2002). Both sexes throughout their lifecycles are transparent, which makes analysis of development and the identification of mutants possible at the cellular level in living preparations, using light microscopy (Riddle *et al.*, 1997). Finally, every cell in the body is accessible to laser microsurgery (Bargmann & Avery, 1995).

With such an extensive understanding of its biology, it is perhaps no wonder that *C. elegans* has been adopted as a "model nematode" for parasitic species. It has certainly been

crucial to identifying and generally understanding the modes of action of several classes of anthelmintic drugs, including the macrocyclic lactones (the avermectins/milbemycins) (Geary & Thompson, 2001). Although *C. elegans* may represent a good model for studying drug action in closely related parasitic species of nematode such as *H. contortus* (Blaxter *et al.*, 1998a), inferences made to describe their activity in distantly related species, such as the filaria, must be treated with more caution (Geary & Thompson, 2001). For example, although inhibition of feeding and motility appear to be important targets for the macrocyclic lactones in *C. elegans* and *H. contortus* (Kass *et al.*, 1980; Avery & Horvitz, 1990; Gill *et al.*, 1991, 1995; Geary *et al.*, 1993; Arena *et al.*, 1995; Paiement *et al.*, 1999), in adult filariae, fecundity may be the most important biological process affected by these drugs (Awadzi *et al.*, 1985; Duke *et al.*, 1991; Lok *et al.*, 1995).

1.5 Nematode anatomy

Nematodes, despite their diversities in habitat, lifestyle and size, share considerable similarities in both morphology and anatomy. Their basic body plan is that of two concentric tubes separated by a fluid-filled cavity, termed the pseudocoelom. Major components of outer tube include the cuticle, hypodermis, body musculature and the nervous system whereas the inner tube contains the digestive system (including the pharynx and intestine). In adult worms, the pseudocoelomic space houses the gonad (White, 1988). The digestive tract and pseudocoelomic space are under considerable hydrostatic pressure, maintained by osmoregulation probably carried out within the intestine. One function of this hydrostatic "skeleton" is to maintain the worm-like morphology of the nematode in the face of external forces (e.g. surface tension) that might otherwise flatten it (Avery & Thomas, 1997). Below is a description of selected aspects of nematode anatomy; the nervous system is described in section 1.6.

Surrounding the outer tube of the nematode is the cuticle, which is a complex layered structure, composed mainly of collagenous material. The cuticle plays a crucial role in the maintenance of morphology, protection from and/or interaction with the external environment and motility. The proteins that constitute the cuticle are secreted by the underlying hypodermis, the external epithelium. During larval moults the cuticle is shed and replaced in most species by one that varies in chemical composition and ultrastructure,

although this appears not to be a requirement related to growth (Kramer, 1997). In parasitic species, it has been suggested this may be a way of adapting to the different environments faced by the various developmental stages. For example, the cuticle may play a role in evasion or modulation of the host's immune system during invasive stages of the parasite's lifecycle (Blaxter & Bird, 1997; Page, 2001).

The nematode's body musculature is attached to the cuticle via the hypodermis. The body wall muscle cells are arranged into four strips (two dorsally and two ventrally) that run the length of the whole worm. Co-ordinated contraction and relaxation of these muscle strips generates the characteristic sinusoidal movement of nematodes in the dorsal-ventral plane. Hence, *C. elegans* observed on agar plates in the lab are moving on their lateral side. The muscles are able to generate this movement by working against the internal hydrostatic pressure and the cuticle (White, 1988). The orientation of muscles in the head is somewhat different, permitting movement in the lateral as well as dorsal-ventral plane. This is important given the head contains an abundance of sensory neurons that monitor the external environment (Driscoll & Kaplan, 1997).

The inner tube contains the digestive system. This consists of the intestine, which runs the length of the worm and two elaborate muscle complexes at either end that control the flow through it. This is necessary as the intestine is devoid of surrounding muscle cells and thus is mechanically passive, although the muscles involved in locomotion can be exploited to influence the process. At the anterior end of the worm is the pharynx, the muscular organ by which the worms feed, to greater or lesser degrees. The rhythmic pumping of the pharynx enables food to be ingested and then crushed before being forced against the internal hydrostatic pressure into the intestine. Absorption of nutrients within the intestine is rapid. It is thought they are distributed to other parts of the worm via the pseudocoelomic fluid. The muscles at the posterior end of the intestine, surrounding the anus, periodically open to allow defecation, which is driven by the internal hydrostatic pressure. The pharynxes of different species vary considerably in both their morphology and importance to feeding. *C. elegans* is a free-living nematode and a filter feeder, and thus derives the majority of its nutrients via pharyngeal pumping. Hence, the pharynx of this species has a very muscular design (Avery & Thomas, 1997). In contrast, adult filaria appear to only have limited gut function and are thought to obtain a large proportion of their essential

nutrients via transcuticular absorption. This is reflected in the considerably less muscular design of the filarial pharynx (Geary *et al.*, 1995).

1.6 The nematode nervous system

Our understanding of nematode neurobiology has largely been the result of studies conducted on *C. elegans* and *Ascaris suum*, a gut parasite found in pigs that is closely related to *A. lumbricoides*. The similarities observed between the two species has lead some to infer that the nervous system is highly conserved in both anatomy and function across the phylum. Nematode neurons are usually quite simple in morphology, many being monopolar with a single unbranched process although this is not always the case. In addition, strict classification of these cells as motor neurons, interneurons or sensory neurons has proved difficult as many perform two (or more) of these functions. As in vertebrates however, nematode neurons are able to form both chemical and electrical (gap junctions) synapses. The structures though of these synapses are unusual: synaptic connections between neurons are made *en passant* whereas at neuromuscular junctions (NMJs) it is the muscle cells and not the neurons that extend processes to form the synapes (Chalfie & White, 1988; Rand & Nonet, 1997).

Although only composed of a relatively small number of component neurons (in *C. elegans* hermaphrodites there are 302; *A. suum* has 298), the nervous system is the most complicated organ in nematodes. It is composed of two near autonomous units, the pharyngeal and somatic (extrapharyngeal) nervous systems. The pharyngeal nervous system is comprised of approximately 20 neurons all of which, as the name suggests, are located in the pharynx and are responsible for its pumping action (Chalfie & White, 1988). Indeed, if the pharynx of *C. elegans* is separated from the rest of the body pumping continues for sometime afterwards indicating the myogenic nature of this system (Avery & Horvitz, 1989). The somatic nervous system is connected to its pharyngeal counterpart by a pair of neurons, which in *C. elegans* are termed the RIP cells, thus enabling communication between the two.

In the somatic nervous system neuronal processes generally run in bundles orientated longitudinally as cords or circumferentially as commissures. The major areas of neuropil

are the nerve ring, which is located in the anterior of the worm surrounding the pharynx, and the ventral cord, the major process bundle to emanate from it that runs posteriorly for almost the entire length of the animal. The nerve ring is the major site of signal integration in the animal. It is here that information from the major sensory neurons, located in the head, are collected, processed and acted upon; the emanating signals frequently alter locomotion but may also regulate other processes such as feeding and fecundity. The ventral cord consists of interneuron and motorneuron processes, their cell bodies lying adjacent to these bundles. The other major longitudinal bundle running posteriorly from the nerve ring is the dorsal cord. Motorneuron axons that form NMJs with the ventral muscles run in the ventral cord. Motorneuron axons that form NMJs with the dorsal muscle also originate in the ventral cord; the axons then run circumferentially around the animal to the dorsal midline where they then turn and run either anteriorly or posteriorly to the site where they form their NMJs. The synaptic connections between the motorneurons and either the ventral or dorsal muscles provide the framework for regulating movement. A number of sublateral cords also originate from the nerve ring. The neurons found in the tail regions vary considerably between sexes (Chalfie & White, 1988).

Based again on studies principally conducted in *C. elegans* and *A. suum*, the nematode nervous system has been determined to use a number of classical neurotransmitters including ACh (acetylcholine), GABA (γ-aminobutyric acid), glutamate, 5-HT (5-hydroxytryptamine/serotonin) and other monoamines. It has also been established that nematodes possess an extensive number of neuroactive peptides (reviewed in Brownlee *et al.*, 2000). GABA is the major inhibitory neurotransmitter in nematodes, whereas the major excitatory transmitters are ACh (on to the somatic muscle) and glutamate (in interneurons). Glutamate also functions as an inhibitory neurotransmitter on to pharyngeal muscle, controlling the timing of pharyngeal relaxation (Avery & Thomas, 1997), and as a neuromodulator in the somatic nervous system; ACh also has an inhibitory role in the pharynx. The contrasting roles of glutamate are likely to be achieved via interactions with different ion-selective classes of neurotransmitter receptors. For example, the excitatory role in the somatic nervous system is conveyed by the cation-selective GLR (non-NMDA type glutamate receptor) and NMR (NMDA-type glutamate receptor) glutamate receptors (Zheng *et al.*, 1999). The inhibitory role in the pharynx is also most certainly due to interaction with glutamate-gated chloride channels (GluCls) (Martin, 1996; Avery &

Thomas, 1997; Pemberton *et al.*, 2001) whereas the modulatory roles may involve both classes of receptor.

1.7 *D. immitis* chemotherapy

Treatment strategies exist to both prevent and cure *D. immitis* infections. The approaches briefly described here are derived from guidelines issued by the American Heartworm Society (Knight, 1998), although the drugs detailed are commonplace globally. Chemoprophylaxis is the most effective way of controlling heartworm disease. The timing and duration of the treatment coincides with the transmission season, which effectively means the warmer months of the year when mosquito populations are sustained. The major drugs used in preventative treatment are members of the macrocyclic lactone class (avermectin/milbemycins) of anthelmintics. These compounds are discussed in more detail in later sections as they target the GluCls, the focus of this study. The macrocyclic lactones licensed in the USA as chemoprophylactics are ivermectin (IVM), milbemycin oxime, moxidectin and selamectin. These compounds are extremely effective at killing larval stages of heartworm although are less effective against adult worms, hence their primary use as chemoprophylactics. One advantage of these drugs is that they only need be administered monthly with certain members of this group exhibiting impressive retro-activities; an added bonus if a dose is missed. An alternative to these drugs is diethylcarbamazine (DEC). However, if not taken every day DEC provides little protection against infection making the macrocyclic lactones a much more attractive option. The macrocyclic lactones also generally provide a greater margin of safety compared to DEC, although in certain breeds of dogs, notably collies, these drugs can have a neurotoxic effect. This is because collies are homozygous for a mutated version of the *mdr1* gene, which encodes P-glycoprotein (P-gp), a transmembrane protein pump found in the blood brain barrier; the mutant alleles encode a truncated protein (Mealey *et al.*, 2001). IVM is a substrate for P-gp, therefore in dogs expressing the truncated form the drug accumulates in the central nervous system. It may then reach concentrations that have been shown to interact with other neurotransmitter receptors *in vitro*, probably explaining the neurotoxicity observed (Krause *et al.*, 1998; Khakh *et al.*, 1999; Adelsberger *et al.*, 2000; Dawson *et al.*, 2000; Shan *et al.*, 2001).

Should an animal be already infected with heartworms the first step is usually to remove the adult worms. Although it has been recently shown that IVM may have a partial adulticidal effect over long treatment periods (Rawlings, 2002), removal of the adults is usually achieved by surgery, in very severe cases, or by the administration of arsenical compounds (melarsomine or thiacetarsemide). Treatment of heartworm with either of these drugs is risky for the dog. During this period the animal is carefully monitored for toxic side effects of the drugs and for pulmonary thromboembolism, an unfortunate but frequent outcome caused by the dead or dying adult worms. Once the removal of the adult heartworms has been established and the dogs have recovered from the toxic side effects of the arsenical compounds removal of the microfilariae may begin. Although no drugs are officially licensed as microfilaricides, in the US at least, extra-label use of certain drugs is permitted. In this instance the macrocyclic lactones, particularly IVM and milbemycin oxime, are extremely effective and frequently used. The rapid death on mass of microfilaria can promote a toxic reaction in the dog and for a short duration after treatment the dogs again must be carefully monitored. The prolonged used of macrocyclic lactones drugs should then remove further larval stages within the infected animal (Knight, 1998).

1.8 The avermectins/milbemycins (the macrocyclic lactones)

1.8.1 Introduction

The avermectins (AVMs) and milbemycins are structurally related classes of 16-membered macrocyclic lactones that possess potent anthelmintic, insecticide, and acaricide properties. Both were isolated in the 1970's from soil-dwelling *Streptomyces* species and remain at the forefront of control for a range of endo- and ecto-parasites, including nematodes. However, they have little effect on tapeworms or flukes. A feature of these compounds is their low toxicity towards host species. The first commercial member of the AVMs/milbemycins (AMs), ivermectin (IVM), was introduced in 1981. IVM (22,23-dihydroavermectin B_1a) is a semi-synthetic derivative of avermectin B_1a, the most potent of the 8 natural AVMs originally isolated (Chabala *et al.*, 1980). Like the other AMs, IVM has a broad spectrum of activity and has been widely used to combat nematode parasitism of humans, their livestock and other domesticated animals, and crops (Campbell, 1989). In man IVM has become the cornerstone of the mass drug administration program to eradicate onchocerciasis (river blindness) (Meredith & Dull, 1998) and is used in part for a similar program to eliminate

lymphatic filariasis (Brown *et al.*, 2000). Given the similarity in structure and action between the AVMs and milbemycins, it is often assumed that data obtained with one member of the family is generally applicable to all. Though this may be true, it is probably sensible to note that this may not always be the case. Therefore, a point is made in the following sections to note which compounds were used in the data reviewed.

1.8.2 Biological effects

A number of *in vitro* studies have shown that the AMs inhibit pharyngeal pumping (and hence feeding), motility and fecundity in susceptible nematodes. In *H. contortus,* pharyngeal pumping is the most sensitive of the processes affected by these anthelmintics (Gill *et al.*, 1991, 1995; Geary *et al.*, 1993; Paiement *et al.*, 1999). IVM concentrations ≥0.1nM paralyse the pharynx of adult *H. contortus*, whereas a reduction in motility leading to overall paralysis requires ≥10nM IVM. Interestingly paralysis is confined to the mid-body region of the worm, with the head and tail regions still able to move normally (Geary *et al.*, 1993). AVMs exert similar effects at comparable concentrations on *H. contortus* larvae (Gill *et al.*, 1991, 1995) and *C. elegans* (Kass *et al.*, 1980; Avery & Horvitz, 1990; Arena *et al.*, 1995). In filaria, as observed for *D. immitis*, the AMs appear to very effective at killing larval stages of the parasite, which presumably occurs through inhibition of one of these processes. Microfilariae do not possess a functioning intestine (Bryant, 1988) and so overall paralysis seems the most likely way that these drugs kill this larval stage. The most important effect on the adult filaria appears to be suppression of new microfilariae production (Awadzi *et al.*, 1985; Duke *et al.*, 1991; Lok *et al.*, 1995), as the AMs are not particularly effective at killing the resident macrofilariae; similar effects on fecundity are seen in *H. contortus* and *C. elegans* (Le Jambre *et al.*, 1995; Grant, 2000). It remains unclear which mechanism is responsible for the overall potency of the AMs *in vivo* towards parasitic nematodes, but it seems likely that the relative importance of each of these major effects may differ between species (Gill & Lacey, 1998) and even developmental stages.

1.8.3 Molecular targets of the AMs

The major sites of action of the AMs appear to be the GluCls, although as discussed below, other molecular targets may also be important to mediate some of their biological effects. It

was apparent from early experiments that the AVMs interacted with ligand-gated chloride channels from both target and non-target species (Kass *et al.*, 1980; Supavilai & Karobath, 1981; Graham *et al.*, 1982; Pong & Wang, 1982). It was therefore not surprising that it was thought that their main target could be the GABA receptor present at the nematode neuromuscular junction, though micromolar concentrations were required to elicit the blocking effects observed (Holden-Dye & Walker, 1988; Holden-Dye & Walker, 1990). A patch-clamp study by Martin and Pennington (1989) on *A. suum* muscle also demonstrated that IVM antagonised GABA receptors, reducing the single-channel conductance and the frequency of channel openings. The same study showed that IVM activated both non-GABA chloride channels on outside-out patches and 'noisy' cation-selective channels on inside-out patches. Both channels remain poorly characterised and have not been observed in other preparations (Holden-Dye & Walker, 1990). Older studies have also indicated that AVM B_{1a} could also block transmission between interneurons and excitatory motorneurons in the ventral cord and ventral inhibitory neuromuscular transmission (Kass *et al.*, 1980; Kass *et al.*, 1984). Delany *et al* (1998) proposed that this could be explained by the presence of GluCls on motor neurons innervating the somatic muscles. Again the concentrations of AVMs used were in the micromolar range, significantly higher than those found to exert anthelmintic effects *in vivo* or *in vitro*. Today it is clear that the AMs can interact with a wide variety of channels, including mammalian glycine and $GABA_A$ receptors (Adelsberger *et al.*, 2000; Dawson *et al.*, 2000; Shan *et al.*, 2001), α7 nicotinic acetylcholine receptors (nAChRs) (Krause *et al.*, 1998) and $P2X_4$ receptors (Khakh *et al.*, 1999). However, the AMs low toxicity towards most mammalian species suggests that they do not normally interact with these latter receptors; they probably do not reach the required concentrations to elicit the observed *in vitro* effects, as the therapeutic doses required to eliminate the parasites are low and the compounds are constantly removed from the host's central nervous system by P-glycoproteins (Burkhart, 2000).

The first direct evidence for an AM interaction with GluCls was revealed in insect muscle. IVM was shown to activate a chloride channel, which was sensitive to the glutamate analogue ibotenate but insensitive to GABA (Duce & Scott, 1985). In nematodes, IVM was shown to bind a site in membranes prepared from *C. elegans* with a 100-fold higher affinity than binding to membranes from rat brain. The relative binding affinities of IVM and a number of derivatives were shown to correlate well with their ability to inhibit motility in

C. elegans, i.e. the higher the binding affinity the more potent the effect on motility (Schaeffer & Haines, 1989). High affinity binding by a series of AM analogues has also been demonstrated to membranes prepared from *H. contortus* (Rohrer *et al.*, 1994; Hejmadi *et al.*, 2000; Cheeseman *et al.*, 2001). When avermectin-sensitive chloride currents were produced in *Xenopus* oocytes injected with *C. elegans* mRNA, they were found to be GABA-insensitive (Arena *et al.*, 1991) but, later, to be sensitive to glutamate (Arena *et al.*, 1992). This, and the subsequent cloning of cDNAs encoding avermectin sensitive glutamate-gated chloride channel (GluCl) subunits from *C. elegans*, parasitic nematodes and insects (Cully *et al.*, 1994; Cully *et al.*, 1996b; Dent *et al.*, 1997; Laughton *et al.*, 1997a; Forrester *et al.*, 1999; Jagannathan *et al.*, 1999; Dent *et al.*, 2000; Cheeseman *et al.*, 2001; Horoszok *et al.*, 2001; Forrester *et al.*, 2002; Forrester *et al.*, 2003) has led to the belief that the GluCls are the principal targets through which the AMs exert their biological effects on nematodes. Electrophysiological studies of pharyngeal preparations from both *C. elegans* and *A. suum* have shown that the AMs act on GluCls to inhibit pumping (Martin, 1996; Adelsberger *et al.*, 1997; Pemberton *et al.*, 2001), though Brownlee *et al* (1997) also suggested a possible interaction with GABA receptors in the *A. suum* pharynx. In insects the situation has been recently complicated by the suggestion that the *Drosophila* avermectin receptor may contain both the GluCl subunit and the GABA receptor subunit, RDL (Ludmerer *et al.*, 2002) and by the discovery of avermectin-sensitive histamine-gated chloride channels (HisCls) in this species (Gisselmann *et al.*, 2002; Zheng *et al.*, 2002). Mutations in the *ort* gene, which encodes one of the HisCl subunits, alter the susceptibility of the flies to the avermectins (Georgiev *et al.*, 2002; Iovchev *et al.*, 2002) suggesting that these channels may be important targets *in vivo*. In nematodes, where no HisCls have been demonstrated, the evidence remains strongly in favour of the GluCls as the clinically relevant AM target.

1.9 The glutamate-gated chloride channels

1.9.1 Molecular biology

The initial isolation of the *C. elegans* GluClα1 and GluClβ cDNAs by the Merck group (Cully *et al.*, 1994) showed that the GluCls are members of the ligand-gated ion channel (LGIC) superfamily, closely related to the $GABA_A$ and glycine receptors. The individual subunit sequences show all the characteristics of this family, with a long N-terminal

extracellular domain followed by four membrane-spanning domains (Ortells & Lunt, 1995). There is a long intracellular loop between the third and fourth of these membrane-spanning domains that contains consensus sequences for protein kinases: in other members of the family protein phosphorylation has been shown to affect the channel properties (Porter *et al.*, 1990; Wafford & Whiting, 1992; Leidenheimer *et al.*, 1993; Krishek *et al.*, 1994; McDonald & Moss, 1994, 1997; Moss *et al.*, 1995, 1996; Macdonald, 1995; McDonald *et al.*, 1998; Filippova *et al.*, 2000). By analogy with other ligand-gated ion channels, the mature receptor is presumed to be a pentamer, made up of different types of subunit and with the second membrane-spanning domains lining the ion pore. This presumption is largely based on biochemical and structural studies conducted on nAChRs (reviewed in Karlin, 2002).

Further cloning studies, together with the complete *C. elegans* genomic sequence, have led to the identification of a small family of six GluCl genes in the model nematode (table 1.1). At least two of these genes, *avr-14* and *avr-15*, are alternatively spliced (Dent *et al.*, 1997; Laughton *et al.*, 1997a; Vassilatis *et al.*, 1997a) to yield at least eight possible subunits. Since the native GluCls are presumed to be pentamers, it is clear simply from the number of GluCl subunit genes that multiple forms of this channel must exist in worms, and there are therefore likely to be multiple AM target sites. To date there is no direct evidence on the subunit composition of any nematode GluCl. The parasitic species about which we have most information is *H. contortus*. So far, three genes encoding four GluCl subunits have been identified in this organism (table 1.1) (Delany *et al.*, 1998; Forrester *et al.*, 1999; Jagannathan *et al.*, 1999; Cheeseman *et al.*, 2001). Three of the *H. contortus* subunits are clearly orthologues of ones in *C. elegans,* the HcGluClβ and α3 subunits; in the absence of a complete genome sequence it is impossible to know whether or not orthologues of the other *C. elegans* genes await discovery. HcGluClα does not seem to be orthologous to any of the *C. elegans* genes (Cheeseman *et al.*, 2001), indicating that there are differences in the genetics of the GluCls between even quite closely related nematode species: *H. contortus* and *C. elegans* are in the same clade of the phylogenetic tree proposed by Blaxter *et al* (1998a). Before commencing this project partial sequences corresponding to *avr-14* had also been isolated from *A. suum* and the filarial species *O. volvulus* and *D. immitis* (Cully *et al.*, 1996a): the alternative splicing of this gene is conserved in all the nematodes where this

has been examined (Jagannathan *et al.*, 1999). The final member of the *C. elegans* GluCl family, *glc-4*, is somewhat divergent from the α and β subunits and may therefore represent a 'γ' subunit. Partial GluCl cDNAs have also been discovered in *Onchocerca ochegi* (a cattle parasite) and more recently *Brugia malayi*.

C. elegans **gene**	***C. elegans*** **subunit**	***H. contortus*** **gene**	***H. contortus*** **subunit**	**Other nematode GluCl partial cDNAs**
glc-1	GluClα1			α-type EST for *O. ochengi*
glc-2	GluClβ	*Hcglc-2* *(hg4)*	HcGluClβ (HG4)	β-type EST for *O. ochengi*
glc-3	GluClα4			
glc-4				Partial *glc-4* cDNA
avr-14 (*gbr-2*)	GluClα3A (GBR-2A) GluClα3B (GBR-2B)	*Hcavr-14* *(gbr-2)*	HcGluClα3A (HcGBR2A) HcGluClα3B (HcGBR2B)	Partial *avr-14* cDNAs for *D. immitis, O. volvulus & A. suum.*
avr-15	GluClα2A GluClα2B			
		HcGluClα	HcGluClα	

Table 1.1 Nematode GluCl genes and subunits identified before commencing the project. The names used for the GluCl genes and subunits are taken from Yates *et al* (in press): other names that have been used in the literature are given in brackets.

1.9.2 GluCl pharmacology

Our current understanding of nematode GluCl pharmacology has been derived from a number of experimental approaches: two-electrode voltage clamp (TEVC) recordings from *Xenopus* oocytes mainly expressing *C. elegans* GluCl subunits (see table 1.2 for summary), radioligand binding studies on membranes from either whole worms or cell-lines expressing GluCl subunits (see table 1.3 for summary), and electrophysiological investigations using nematode tissue preparations. Ivermectin phosphate ($IVMPO_4$) was used in a number of experiments reviewed below instead of IVM due to its increased solubility in water. In subsequent chapters for convenience the distinction is not made between the two compounds.

GluClα1 and GluClβ were the first GluCl subunits to be cloned and expressed in oocytes (Cully *et al.*, 1994). Cells expressing both subunits exhibited responses to applied glutamate and $IVMPO_4$ comparable to those previously reported in oocytes injected with *C. elegans* mRNA (Arena *et al.*, 1991, 1992). Glutamate rapidly activated an inward chloride current that was fully reversible when the ligand was washed out. However, the high EC_{50} value for glutamate (1.36mM) is not comparable to that for oocytes injected with *C. elegans* mRNA (300μM) (Arena *et al.*, 1992), and this, coupled with the lack of any evidence that the two genes are expressed in the same cells, may argue against GluClα1 and GluClβ co-assembly *in vivo*. Partial desensitisation of the response was observed with glutamate at concentrations >1mM. $IVMPO_4$ at high concentrations (>10nM, EC_{50}=190±7nM) also directly activated the chloride current, albeit with a slower onset. The current was essentially irreversible, even when the $IVMPO_4$ was removed, and the channels exhibited little desensitisation. At lower concentrations (<10nM) $IVMPO_4$ was unable to activate the channels but potentiated the glutamate response at sub-maximal glutamate concentrations. GluClα1β heteromers exhibited a distinct pharmacology compared with other LGICs, including the glutamate-gated cation channels. Although activated by ibotenate, a structural analogue of glutamate, GluClα1β were insensitive to NMDA, kainate, quisqualate, AMPA, acetylcholine and muscimol at 1mM and L-aspartate, GABA, glycine, histamine, β-alanine and taurine at 10mM (Cully *et al.*, 1994). Subsequent studies on other GluCl subunits revealed a similar pharmacology (Dent *et al.*, 1997; Vassilatis *et al.*, 1997a; Horoszok *et al.*, 2001). In addition, both glutamate (1mM) and $IVMPO_4$ (1μM) activated currents were partially blocked by 100μM picrotoxin (PTX), a chloride channel blocker, by 68% and 61% respectively. Another chloride channel blocker, flufenamic acid (200μM), exhibited a similar degree of inhibition of the $IVMPO_4$ response (Cully *et al.*, 1994). The concentrations of both compounds (i.e. PTX and flufenamic acid) used were comparable to those used to partially block the chloride currents in oocytes injected with *C. elegans* mRNA (Arena *et al.*, 1992). The IC_{50} values for PTX blockade of the heteromeric channels to glutamate and $IVMPO_4$ were later determined to be 42μM and 52μM respectively (Etter *et al.*, 1999).

Both the GluClα1 and GluClβ subunits are also able to form homomeric channels. GluClα1 homomers were activated by $IVMPO_4$ (EC_{50}=140nM) in typical fashion but were

insensitive to glutamate. Later studies demonstrated that glutamate actually bound to GluClα1 subunits but could not couple binding to gating (Etter *et al.*, 1996). Conversely, GluClβ homomers were activated by glutamate (EC_{50}=380μM) but were insensitive to IVM, either activating the channels or potentiating the glutamate response. PTX was significantly more effective at blocking GluClβ homomers (IC_{50}=77nM) compared to heteromeric channels.

The alternatively spliced GluClα2A and 2B subunits were both able to form homomeric receptors sensitive to both glutamate (EC_{50}=2mM & 208μM respectively), to which they exhibited rapid desensitisation, and IVM or $IVMPO_4$ respectively (GluClα2B $IVMPO_4$ EC_{50}=108nM) (Vassilatis *et al.*, 1997a). GluClα2B was shown to form heteromeric receptors with GluClβ: these heteromeric receptors exhibited a slower desensitisation rate and also had increased sensitivity to glutamate (EC_{50}=62μM) compared with homomeric channels of either subunit. This was in contrast to GluClα1β heteromers, which exhibited decreased sensitivity to glutamate compared with GluClβ homomers. The $IVMPO_4$ sensitivity remained unchanged. PTX weakly blocked the GluClα2B homomers but had no effect at 100μM on GluClα2Bβ heteromers (Vassilatis *et al.*, 1997a).

Of the other subunits GluClα3B formed homomeric channels that were glutamate- (10mM) and IVM- (10μM) sensitive (Dent *et al.*, 2000). The alternatively spliced subunit GluClα3A did not respond to either agonist. It is not known whether this is due to a lack of sensitivity or the inability of this subunit to associate into homomeric channels. The GluClα4 subunit was also able to form homomeric receptors that responded to both glutamate (EC_{50}=1.9mM) and IVM (EC_{50}=400nM) (Horoszok *et al.*, 2001). Although PTX insensitive, the GluClα4 channels were blocked by BIDN (IC_{50}=200nM) and fipronil (IC_{50}=11.5μM), compounds that have been demonstrated to act at insect GABA-gated chloride channels (Hosie *et al.*, 1995; Rauh *et al.*, 1997; Hamon *et al.*, 1998).

Recently the first GluCl subunit from a parasitic nematode, HcGluClα, has been expressed in oocytes. This subunit forms homomeric channels that are gated by glutamate (EC_{50}=8.4μM) and IVM (EC_{50}=131nM). In addition, potentiation of the glutamate response

appeared to be observed by pre-application of 10nM IVM but was deemed not to be statistically significant (Forrester *et al.*, 2003).

The pharmacology, described above, clearly distinguishes the GluCls from other members of the LGIC superfamily and notably, the cationic glutamate receptors. It remains difficult however to comment expansively and draw firm conclusions on the quantitative responses elicited by glutamate and other ligands, as it appears likely that not all of these subunits form homomeric channels *in vivo*. In fact, as with other classes of LGICs, it is plausible that most native GluCls are heteromers. These receptors may well have distinct quantitative (and possibly qualitative) differences in their responses to various ligands compared to that of homomers comprised of the heteromers' constituent subunits. In addition, rates of desensitisation, a key process in regulating the activity of LGICs, may be significantly altered in these heteromers compared to the homomers. As described above there is some evidence to support such statements in GluCls (Cully *et al.*, 1994, Vassilatis *et al.*, 1997a). Perhaps to ascertain a more accurate quantitative profile of native GluCl pharmacology, further studies using TEVC and the *Xenopus* oocyte expression system should focus on subunit combinations, based on localisation studies that are likely to exist *in vivo*.

The concentrations of IVM required to gate *C. elegans* GluCls, expressed in *Xenopus* oocytes are high when compared to the concentrations able to paralyse pharyngeal pumping and motility in worms *in vitro*. However a recent electrophysiological study demonstrated that the native pharyngeal GluCls from *C. elegans* were considerably more sensitive to IVM than GluCls expressed in *Xenopus* oocytes (Pemberton *et al.*, 2001). Glutamate applied to the pharynx elicited a rapidly desensitising, reversible, chloride-dependent depolarisation. An EC_{50}=166μM was within the range observed for subunits expressed in oocytes. Interestingly the inhibitory response observed was caused by a depolarisation of the membrane and not the previously suggested hyperpolarisation (Dent *et al.*, 2000; discussed below). IVM also elicited a chloride-dependent depolarisation of the muscle, however the response was typically irreversible and highly potent, with an EC_{50}=2.7nM (Pemberton *et al.*, 2001). The potency of the IVM response was comparable with concentrations shown to paralyse the pharynx *in vitro* (Avery & Horvitz, 1990; Geary *et al.*, 1993). This could be explained by potentiation of the IVM response by endogenous glutamate (Pemberton *et al.*, 2001). In support of this, a recent study has shown that

glutamate potentiated AM binding to the *H. contortus* HcGluClα subunit (Forrester *et al.*, 2002). Alternatively the differing IVM sensitivities could be explained by the native subunit composition. As outlined above, so far only a limited number of subunit combinations have been successfully expressed in oocytes and none of these may reflect the composition of native GluCl.

The depolarisation of the pharyngeal muscle described above, i.e. when the membrane potential becomes less negative than the resting potential of the cell, is due to the relative local chloride ion concentrations within and outside the cells. In this instance, the intracellular chloride ion concentration appears to be greater than the extracellular concentration. Therefore when the GluCls are opened, by application of glutamate or IVM, chloride ions flow down their concentration gradient out of the pharyngeal muscle cells causing them to become less negative and hence, depolarised (Pemberton *et al.*, 2001). Other studies have shown that glutamate and AM application to pharyngeal muscle and glutamate application to motor neurons, both preparations from *A. suum*, elicit hyperpolarizing currents (Martin, 1996; Davis, 1998). In these cases the relative local intra- and extracellular chloride ion concentrations are reversed. Hence, when the GluCls are activated the chloride ions flow down their concentration gradient into the cells. Therefore, the effect of glutamate and IVM via GluCls (i.e. to elicit a depolarising or hyperpolarizing currents) is dependent on the local relative chloride ion concentration, which may vary between species and indeed regions of the animal itself.

Studies of *H. contortus* GluCl pharmacology have largely taken a radioligand binding assay approach. IVM has been shown to bind a high affinity site in *H. contortus* whole worm membrane preparations with a K_D ranging from 0.07-0.6nM (Rohrer *et al.*, 1994; Gill & Lacey, 1998; Hejmadi *et al.*, 2000; Cheeseman *et al.*, 2001) depending on the experimental conditions. This is comparable to the high affinity IVM binding site (K_D = 0.26nM) described in *C. elegans* (Schaeffer & Haines, 1989) and to the concentrations of the drug that exhibit anthelmintic activity. Further binding studies were conducted with membranes prepared from COS-7 cells, a mammalian cell-line, expressing individual *H. contortus* GluCl subunits (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002). The HcGluClα and HcGluClα3B subunits were shown to bind [^{3}H]-IVM with K_D values of 26-110pM and

70pM respectively. These data implicate the high-affinity IVM binding site to be a GluCl. The HcGluClα3B K_D value was very similar to that for [^{3}H]-IVM at L_3 membrane preparations (Cheeseman *et al.*, 2001), so this subunit could conceivably represent the major IVM binding-site in this larval stage. Other studies indicating that the HcGluClα subunit was mainly expressed in adult worms support this possibility (Forrester *et al.*, 1999). Binding of [^{3}H]-IVM to the individual subunits and whole worm membranes was inhibited by a variety of AVMs, implying they all bound the same site. However glutamate, GABA and PTX failed to compete for the IVM binding site. The recombinant HcGluClα3A and HcGluClβ subunits exhibited no specific [^{3}H]-IVM binding, as predicted from the properties of their *C. elegans* orthologues expressed in *Xenopus* oocytes. A lower affinity-binding site (K_D= 8.7nM) has also been described in *H. contortus* L_3 membranes but the importance of this remains unknown and uncharacterised in molecular terms (Gill & Lacey, 1998).

From the experiments described above, the AMs appear to act by increasing the open probability of the GluCls through either direct activation and/or maybe potentiation of the channels (discussed in chapter 6). This appears to lead to either an essentially irreversible (partly explained by their high affinity interaction) hyperpolarisation or depolarisation of the target cell, be it neuron or muscle. The loss of excitability within in these cells presumably explains the paralysis observed.

GluCl subunit	EC_{50} for glutamate	Hill coeff. glutamate	EC_{50} for IVM	Hill coeff. IVM	Reference
C. elegans m RNA	300μM	1.7	90nM*	1.1	(Arena *et al.*, 1991, 1992)
CeGluClα1	No response to 1mM	-	140nM	1.5	(Cully *et al.*, 1994)
CeGluClβ	380μM	1.9	No response to 1μM*	-	(Cully *et al.*, 1994)
CeGluClα1β	1.36mM	1.7	190nM	2.5	(Cully *et al.*, 1994)
CeGluClα2A	2.0mM	1.5	Response to 10μM	No data	(Dent *et al.*, 1997)
CeGluClα2B	208.3μM	2.1	108nM*	1.6	(Vassilatis *et al.*, 1997a)
CeGluClα2Bβ	62μM	2.4	103nM*	1.85	(Vassilatis *et al.*, 1997a)
CeGluClα3A	No response to 10mM	-	No response to 10μM	-	(Dent *et al.*, 2000)
CeGluClα3B	Response to 10mM	No data	Response to 10μM	No data	(Dent *et al.*, 2000)
CeGluClα4	1.9mM	1.5	400nM	4.9	(Horoszok *et al.*, 2001)
C. elegans pharynx	166μM	No data	2.7nM	No data	(Pemberton *et al.*, 2001)
HcGluClα	8.4μM	2.13	131nM	1.15	(Forrester *et al.*, 2003)

Table 1.2. Summary of the pharmacological properties of GluCl channels expressed in *Xenopus* oocytes. The prefixes Ce- and Hc- denote *C. elegans* and *H. contortus*. Asterisks indicate where $IVMPO_4$ was used instead of IVM. The GluCl described in the pharynx of *C. elegans* is included as a comparison to the data obtained from oocyte recordings.

Membrane prep	K_D [^{3}H]-IVM	Reference
C. elegans membranes	260pM	(Schaeffer & Haines, 1989)
H. contortus L_3 membranes	130pM	(Rohrer *et al.*, 1994)
H. contortus L_3 membranes	110pM, 8.7nM	(Gill & Lacey, 1998)*
H. contortus L_3 membranes	600pM	(Hejmadi *et al.*, 2000)
H. contortus L_3 membranes	70pM	(Cheeseman *et al.*, 2001)
HcGluClα	26pM	(Cheeseman *et al.*, 2001)
HcGluClα3B	70pM	(Cheeseman *et al.*, 2001)
HcGluClα	126pM	(Forrester *et al.*, 2002)

Table 1.3. Summary of the binding affinities displayed by [^{3}H]-IVM to whole nematode membranes and individual GluCl subunits discussed in the text. Binding studies for the *H. contortus* GluCls were conducted with membranes prepared from COS-7 cells, expressing the individual subunits. In the study of Cheeseman *et al.* (2001) no specific [^{3}H]-IVM binding was detected to membranes prepared from COS-7 cells previously transfected with either HcGluClβ or HcGluClα3A plasmid DNA. *Gill & Lacey (1998) have described two distinct [^{3}H]-IVM binding sites, one a high affinity site, which may be attributed to the GluCls and a second lower affinity that remains poorly characterised in molecular terms.

1.9.3 Distribution of nematode GluCl subunits

A full understanding of the functions of the GluCls and the effect of the AMs requires that we know where the receptors are expressed and their subunit composition. In *C. elegans*, reporter gene constructs have shown that *avr-14* and *avr-15* are widely expressed in the nervous system (Dent *et al.*, 1997; Dent *et al.*, 2000) whereas *glc-2* expression is confined to pm4 pharyngeal muscle cells (Laughton *et al.*, 1997b). *Avr-15* is also expressed in the pm4 and pm5 muscle cells of the pharynx (Dent *et al.*, 1997), which is consistent with the IVM effect observed in the pharynx of *C. elegans* being dependent on GluClα2 (Pemberton *et al.*, 2001). It is likely that the pharyngeal receptor is a heteromer comprised of GluClα2, GluClβ and possibly one other GluCl subunit. To date, there have been no reported studies on the expression patterns of the *glc-1*, *glc-3* or *glc-4* genes.

Localisation in *H. contortus* has largely been derived from studies using affinity-purified anti-subunit antibodies in immunofluorescence experiments. HcGluClβ was detected on motor neuron commissures but not on pharyngeal muscle (Delany *et al.*, 1998). Recently, using a different anti-HcGluClβ antibody, this was confirmed, but expression of this subunit was also localised to the lateral and sub-lateral nerve cords. HcGluClα has also

been localised on motor neuron commissures and double immunolabelling experiments showed that the α- and β-subunits co-localise to the same commissures in adult worms (Portillo *et al.*, 2003), strongly suggesting that these subunits are part of the same receptor. Nematodes possess distinct excitatory and inhibitory motor neurons that release acetylcholine or GABA, respectively, at neuromuscular junctions (Stretton *et al.*, 1985; Walrond *et al.*, 1985). The anti-HcGluClα subunit fluorescence overlapped with that produced by an anti-GABA antibody, suggesting that an 'α+β' containing receptor is expressed on inhibitory motor neurons.

HcGluClα3A and α3B, were detected in motor neuron commissures, nerve cords and the nerve ring (Jagannathan *et al.*, 1999), but the antibody used in these experiments did not discriminate between the two subunits. Recently, specific antibodies to the alternatively spliced forms have shown that both HcGluClα3 subunits are expressed in motor neuron commissures but that, outside of these cells, they have different patterns of expression. HcGluClα3B, but not HcGluClα3A, is expressed in nerve cords. Interestingly both subunits are expressed in the head of the worm. HcGluClα3A was found in a pair of lateral neurons, probably sensory amphid neurons and HcGluClα3B to three possible pharyngeal neurons (Portillo *et al.*, 2003). In *C. elegans*, *avr-14* is also expressed in extrapharyngeal neurons in the head of the worm as well as in other sensory neurons (Dent *et al.*, 2000). The expression of the HcGluClα3B subunit in pharyngeal neurons might explain the paralytic effect of the AMs on pharyngeal pumping (Geary *et al.*, 1993). The significance of the expression observed in likely sensory neurons is unknown.

The distribution of the known GluCl subunits in *H. contortus* and *C. elegans* therefore seems to correlate well with the observed actions of the AMs (Avery & Horvitz, 1990; Geary *et al.*, 1993), providing further evidence that these receptors are the main drug targets in nematodes. One exception to this may be the effects of the anthelmintics on fecundity, as there is as yet no direct evidence for any role for the GluCls in the reproductive system. However, it is clear that the distribution of orthologous GluCl subunits is not completely conserved between even closely related species of nematode, i.e. *C. elegans* and *H. contortus*. It may therefore be difficult to draw many conclusions from

these studies regarding subunit expression, and hence function, for even orthologous GluCl subunits in more distantly related species, such as the filaria.

1.10 Project aims

The overall objective of the project was to establish potential molecular targets for the AMs in *D. immitis*. It was hoped that this would further our understanding as to why the AMs are so effective at eradicating larval forms of *D. immitis* and yet are so ineffective at killing the adult worms. Therefore the first aim of the project was to amplify candidate GluCl cDNAs from *D. immitis* mRNA, the known partial *avr-14* cDNA serving as a starting point from which to design oligonucleotide primers (chapter 3). Using degenerate PCR it was hoped that further GluCl cDNAs would be amplified. Cloned GluCl subunits cDNAs were then to be expressed in a system that would enable their pharmacology to be investigated (chapters 4 and 5).

Chapter 2

Materials and Methods

2.1 General materials

2.1.1 Molecular biology reagents

Molecular biology grade chemicals were purchased from BDH chemicals (Poole, UK), Sigma Chemical Company Ltd (Poole, UK) or Fisons Scientific Equipment (Leicestershire, UK) except agarose, which was purchased from Life Technologies (Paisley, UK). Ethidium bromide, DEPC, 30% (v/v) hydrogen peroxide solution, 37% (w/v) formaldehyde solution and formamide were all supplied from Sigma.

2.1.1.1 General buffer/solution compositions

Buffer/solutions	Components
50x TAE	2M Tris-acetate, pH 8.0; 50mM EDTA.
10x TBE	0.89M Tris-HCl, pH 8.0; 0.89M boric acid; 20mM EDTA.
6x DNA Loading Buffer	0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol; 30% (v/v) glycerol in ddH_2O.
DEPC-ddH_2O	ddH_2O was incubated with DEPC (0.1% v/v) overnight at room temperature with stirring and then autoclaved.
Phenol:chloroform (used for DNA)	Phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 1mM EDTA containing 10mM Tris-HCl, pH 8.0.
Phenol:chloroform (used for RNA)	Phenol:chloroform:isoamyl alcohol (125:24:1), acid equilibrated, pH 4.7.
10x MOPS Buffer (500ml)	20.9g MOPS in 350ml DEPC-ddH_2O, adjusted to pH 7.0 with 2M NaOH; added 10ml 1M sodium acetate pH 7.0, 10ml 0.5M EDTA pH 8.0 and adjusted to 500ml with DEPC-ddH_2O. Sterilised by filtration.
10x Formaldehyde Loading Buffer	0.25% (w/v) bromophenol blue; 10mM EDTA, pH 8.0; 50% (v/v) in DEPC-ddH_2O.

2.1.1.2 Enzymes

T4 DNA ligase and all restriction endonucleases (including the appropriate reaction buffers) were supplied by Promega (Southampton, UK).

Enzyme	Buffer Components (all 1x)
T4 DNA Ligase	30mM Tris-HCl, pH 7.8; 10mM $MgCl_2$, 10mM DTT, 1mM ATP
BamHI	Buffer E: 6mM Tris-HCl, pH 7.5; 6mM $MgCl_2$, 100mM NaCl, 1mM DTT
EcoRI	Buffer H: 90mM Tris-HCl, pH 7.5; 10mM $MgCl_2$, 50mM NaCl
NotI	Buffer D: 6mM Tris-HCl, pH 7.9; 6mM $MgCl_2$, 150mM NaCl, 1mM DTT
PstI	Buffer H: see above.
SacI	Buffer J: 10mM Tris-HCl, pH 7.5; 7mM $MgCl_2$, 50mM KCl, 1mM DTT
SalI	Buffer D: see above.
XbaI	Buffer D: see above.
XhoI	Buffer D: see above.
	Multicore Buffer (used for selected double digests): 25mM Tris-Acetate, pH 7.5 (at 37°C); 100mM potassium acetate, 10mM magnesium acetate, 1mM DTT

2.1.1.3 Plasmids

Plasmid	Genotype and size	Supplier
pCR®-Blunt II-TOPO	Kan^r, 3.5kb	Invitrogen (Groningen, Netherlands)
pcDNA3	Amp^r, 5.4kb	
pcDNA3.1-	Amp^r, 5.4kb	
pCMV/*myc*/cyto	Amp^r, 4.9kb	
PGEM®-T Easy	Amp^r, 3 kb	Promega
pFLAG-CMV-5a	Amp^r, 4.7kb	Sigma
L4440 (pPD129.36)	Amp^r, 3kb	Fire Lab (Carnegie Institute of Washington, Baltimore, USA)

2.1.2 Reagents used for bacteria and *C. elegans* cultivation

Media reagents were supplied by Difco laboratories (East Molesey, UK) and Sigma, whilst glycerol was purchased from BDH (Poole, UK). Ampicilin was purchased from Melford Laboratories Ltd (Chelsworth, UK), whilst kanamycin was supplied by Sigma.

2.1.2.1 Culture media/buffers

Medium	Components (per litre)
LB	10g tryptone, 5g yeast extract, 5g NaCl.
LB agar	LB and 15g Bacto-agar.
SOC	20g tryptone, 5g yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.
TSB	0.5g Tryptone, 0.25g Bacto Yeat Extract, 0.25g NaCl, 5g PEG3350, pH 6.1 with 1M HCl; autoclaved and added 2.5ml DMSO, 0.5ml 1M $MgSO_4$, 0.5ml 1M $MgCl_2$, pH was re-adjusted as before if necessary.
NGM agar	3g NaCl, 2.5g Bacto-peptone, 17g Bacto-agar into 1L ddH_2O, autoclaved and then added 1ml cholesterol (5mg/ml in ethanol), 1ml 1M $CaCl_2$, 1ml 1M $MgSO_4$, 25ml 1M potassium phosphate (pH 6.0).
M9 Buffer	6g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.25g $MgSO_4.7H_2O$.

2.1.2.2 Bacterial strains

E. coli Strain	Genotype	Supplier
XL-1 Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^-$ F$^-$ [*pro*AB$^+$ *lac*I^q *lac*Z ΔM15 Tn*10* (*tet*^{r})]	Stratagene Ltd, Cambridge, UK
TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMA-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu)*7697 *gal*U *gal*K rspL (Str^R) *end*A1 *nup*G	Invitrogen
OP50	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^-$ F$^-$ [*pro*AB$^+$ *lac*I^q *lac*Z ΔM15 Tn*10*]	Stratagene

2.1.2.3 Antibiotic solutions

Antibiotic	Stock concentration	Working concentration
Ampicillin	50mg/ml in ddH_2O	50µg/ml
Kanamycin	50mg/ml in ddH_2O	50µg/ml

2.1.3 Mammalian cell-culture reagents

COS-7 (African green monkey kidney cells) and HEK-293T (human embryonic kidney cells) cell-lines were supplied by the European Collection of Cell Cultures (Salisbury, UK). These were maintained in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% (w/v) Foetal Bovine Serum, 2mM L-glutamine, 100,000 units/0.5l penicillin and 100mg/0.5l streptomycin (all Sigma). Trypsin EDTA was also supplied from Sigma. The chemical reagents required for the calcium phosphate transfection were all supplied from Sigma.

Other cell culture & transfection buffers	Components
PBS (1litre)	8g NaCl; 0.2g KCl; 1.44g Na_2HPO_4; 0.24g KH_2PO_4, pH 7.4
HBS	50mM HEPES, pH 7.0; 140mM NaCl; 1.5mM $Na_2HPO_4 \cdot 2H_2O$

2.1.4 Immunocytochemistry reagents

The anti-flag M2® mouse IgG1 monoclonal antibody was supplied from Sigma (catalogue number F3165) where as the anti-*c-myc* mouse IgG1 monoclonal antibody was purchased from Chemicon (Harrow, UK; catalogue number MAB8864). The Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG was supplied by Jackson ImmunoResearch (West Grove, PA, USA; catalogue number 115-095-003).

2.1.5 *Xenopus* oocyte electrophysiology reagents

All buffer constituents and drugs were supplied by Sigma except for glutamate, which was purchased from USB (Cleveland, USA).

Buffer/solution	Components
OR-Mg	5mM HEPES, pH 7.5; 82mM NaCl; 20mM $MgCl_2$; 2mM KCl
Collagenase (type 1A)	1.5mg collagenase per ml of OR-Mg.
ND96	5mM HEPES, pH 7.5; 96mM NaCl; 2mM KCl; 1.8mM $CaCl_2$; 1mM $MgCl_2$; 5% horse serum; filtered sterilised and then added 2.5mM pyruvate, 50μg/ml gentamycin, 1x antibiotic antimycotic solution.
SOS	5mM HEPES, pH 7.5; 98mM NaCl; 1mM $MgCl_2$.

2.2 Methods

2.2.1 General molecular biology methods

2.2.1.1 Agarose gel electrophoresis

Gels were prepared by dissolving 1% (w/v) agarose in 1x TAE or TBE buffer by boiling. After cooling the molten agarose to 50-60°C ethidium bromide was added (0.5μg/ml final concentration) and the mixture was poured into a horizontal perspex gel apparatus. Once set, the comb was removed and the gel was submerged in a tank of 1x TAE or TBE. The DNA samples (0.1-2μg) and molecular weight markers (either 100bp or 1kb DNA ladder; Promega) were mixed with 6x Loading Buffer, loaded on to the gel and electrophoresed at 100V until the marker dyes had sufficiently migrated. Fluorescent ultraviolet light (UV) was used to visualise the DNA.

2.2.1.2 Gel purification of DNA

DNA was purified from agarose gels by using the QIAquick Gel Extraction Kit (QIAGEN Ltd., Crawley, U.K.). All centrifugations during the procedure were performed at 10,000g for 1 minute at room temperature. The DNA fragment to be purified was excised from the gel and weighed. For every 1 volume of gel, 3 volumes of Buffer QG were added (100mg ~ 100μl). The mixture was incubated at 50°C until the gel completely dissolved (approximately 10 minutes), the sample being vortexed every 2-3 minutes to aid the process. Following the addition of isopropanol (1 volume), the sample was applied to a QIAquick spin column that had been placed in a 2 ml collection tube, and centrifuged. After discarding the flow-through, 750μl of Buffer PE (ethanol containing wash buffer) was added and the tube was re-centrifuged. Again the flow-through was removed and the tube was re-centrifuged to remove any residual ethanol. The column was then placed into a clean 1.5ml microfuge tube and 30μl of sterile ddH_2O was added. Following a 1-minute incubation at room temperature, the DNA was eluted by a final centrifugation step.

2.2.1.3 Restriction endonuclease digestion of DNA

A typical restriction digest comprised 1-5μg plasmid DNA, 3μl 10x Reaction Buffer, 3μl 10x BSA (1mg/ml stock), 5-10 units of each restriction enzyme and sterile ddH_2O up to 30μl. The reactions were incubated for 2-4 hours at 37°C. For double digestions, the enzymatic activities of each restriction endonuclease in the various 10x Reaction Buffers, as determined by the manufacturer, were considered before selecting the appropriate one.

2.2.1.4 Ligation of DNA

Approximately 50-200ng of linearised plasmid DNA was mixed with the DNA (the insert) to be sub-cloned in a molar vector:insert ratio somewhere between 1:1 and 1:3. To the sample was added 1 unit T4 DNA Ligase, 1μl 10X Ligase Buffer and sterile ddH_2O to 10μl. The ligation reactions were performed at 16°C for approximately 16 hours.

2.2.1.5 Preparation of *E. coli* competent cells

XL-1 Blue *E. coli* was used for routine sub-cloning. From one colony of bacteria a small culture (5ml LB) was grown overnight at 37°C with shaking; a sample (1ml) of which was subsequently used to inoculate 100ml LB in a 1-litre flask. The inoculated culture was grown at 37°C with shaking until an A_{600} of 0.5 was reached after which the cells were pelleted by centrifugation at 6,000g for 10 minutes at 4°C. The supernatant was removed and the cells were re-suspended in 10ml of ice cold TSB. After a 10-minute incubation on ice, the re-suspended cells were aliquoted (100μl) and stored at -70°C until required.

2.2.1.6 Transformation of plasmid DNA

A sample of the ligation reaction (5-10μl) was added to a 100μl aliquot of competent XL-1 Blue *E. coli* that had previously been thawed on ice. The cells were carefully mixed by gently swirling with the pipette tip and left to incubate on ice for 5-30 minutes. Following heat shocking at 42°C for 30 seconds, the cells were replaced on ice for a further 2 minutes. SOC (250μl) was then added and the cells were incubated for 1 hour at 37°C with shaking. The transformation mixture was spread (usually 50 and 200μl) on to antibiotic-containing LB-Agar plates, which were incubated overnight at 37°C to enable colony growth.

Colonies were then selected and grown in antibiotic containing LB overnight; the resultant cultures were miniprepped (see below) and restriction digested to identify recombinant clones.

2.2.1.7 Small-scale preparation of plasmid DNA (minipreps)

Small-scale isolation of plasmid DNA was carried out using the CONCERT™ Rapid Plasmid Miniprep System (Gibco BRL, Paisley, U.K.). All centrifugations during the procedure were performed at 12,000g at room temperature.

Buffer	Composition
Cell Suspension Buffer (G1)	50mM Tris-HCl, pH 8.0; 10mM EDTA; 20mg/ml RNase A
Cell Lysis Buffer (G2)	200mM NaOH, 1% (w/v) SDS
Neutralization Buffer (M3)	Contains acetate and guanidine hydrochloride*
Wash Buffer (G4)	Contains NaCl, EDTA, Tris-HCl, pH 8.0, and 75% ethanol*
TE Buffer (TE)	10mM Tris-HCl, pH 8.0; 0.1mM EDTA

*Proprietary formulation

A sample (3-5ml) of an overnight bacterial culture (grown from one colony in antibiotic-containing LB) was centrifuged for 2 minutes. Often the remaining culture was used to make bacterial cell-stocks (300µl culture mixed with 600µl glycerol; stored at -70°C) for long-term storage of useful plasmids. Following centrifugation, the supernatant was removed and the bacterial pellet was re-suspended in 250µl of Cell Suspension Buffer until homogeneous. Cell Lysis Buffer (250µl) was added to the re-suspended cells. The tube's content was mixed carefully by inverting 5 times and then incubated for 5 minutes at room temperature. Neutralization Buffer (350µl) was added to stop the lysis reaction. As before the tube was carefully mixed by 5 inversions before being centrifuged for 10 minutes. The supernatant was loaded into a spin cartridge placed in a 2-ml wash tube and centrifuged for 1 minute. This enabled the DNA to bind the silica-based membrane. Following centrifugation the flow-through was discarded and the spin cartridge was placed back into the wash tube. Wash Buffer (700µl) was added to the cartridge, which was then centrifuged for 1 minute. The flow-through was discarded and the tube was re-centrifuged for 1 minute

to remove any residual Wash Buffer. The spin cartridge was then placed into a clean 1.5ml microfuge tube. Pre-warmed (65-70°C) TE (75µl) was added to the cartridge to elute the DNA, which was recovered following a 1-minute incubation by a 1-minute centrifugation.

2.2.1.8 Large-scale preparation of plasmid DNA (maxiprep)

Large-scale isolation of plasmid DNA was carried out using the QIAGEN Plasmid Maxi Kit (QIAGEN Ltd., Crawley, U.K.).

Buffer	Composition
P1 (Resuspension Buffer)	50mM Tris-HCl, pH 8.0; 10mM EDTA; 100µg/ml RNase A; stored at 4°C.
P2 (Lysis Buffer)	200mM NaOH, 1% (w/v) SDS.
P3 (Neutralization Buffer)	3.0M potassium acetate, pH 5.5.
QBT (Equilibration Buffer)	750mM NaCl; 50mM MOPS, pH 7.0; 15% (v/v) isopropanol; 0.15% (v/v) Triton® X-100.
QC (Wash Buffer)	1.0M NaCl; 50mM MOPS, pH 7.0; 15% (v/v) isopropanol.
QF (Elution buffer)	1.25M NaCl; 50mM MOPS, pH 8.5; 15% (v/v) isopropanol.

A starter culture (2-5ml antibiotic-containing LB), inoculated initially with a single colony of the desired bacterial strain, was grown for ~8 hours at 37°C with shaking. A sample (100µl) of this culture was used to inoculate a 100ml LB in a 500ml flask, which was incubated overnight at 37°C with shaking. The bacterial cells were harvested by centrifugation (6,000g for 15 minutes at 4°C) and the supernatant was removed. After re-suspension in 10mls of Buffer P1, the cells were lysed by the addition of 10mls of Buffer P2; the lysis reaction was gently mixed by 5 inversions and left to proceed for 5 minutes at room temperature. Buffer 3 (10ml) was added to neutralize the lysis reaction; the tube was mixed by inversion 5 times and left to incubate on ice for 20 minutes. The sample was centrifuged at ≥20,000g for 30 minutes at 4°C after which the supernatant was removed and re-centrifuged for 15 minutes. During the centrifugation the QIAGEN-tip 500 (anion-exchange column) was equilibrated by the addition of 10ml OF Buffer QBT. The column was allowed to fully empty by gravity flow before the cell lysate supernatant was applied. After fully emptying, the column was washed twice with 30ml of Buffer QC before 15ml of

Buffer QF was added to elute the DNA. Isopropanol (10.5ml) was added to precipitate the DNA; the sample being mixed and then centrifuged at ≥15,000g for 30 minutes at 4°C. After the supernatant was removed the DNA pellet was washed with room temperature 70% ethanol (5ml) and re-centrifuged at ≥15,000g for 10 minutes at 4°C. The ethanol was removed and the pellet was allowed to air-dry for 5-10 minutes before being re-suspended in 500µl ddH_2O. The yield of the maxiprep was determined using a spectrophotometer as described in the following section.

2.2.1.9 Nucleic acid quantitation by spectrophotometry

Concentration and purity of nucleic acids were determined using a spectrophotometer. Nucleic acids absorb UV light maximally at 260nm enabling direct calculation of the DNA/RNA concentration according to the equation:

Nucleic acid conc. (µg/ml) = A_{260} x dilution factor x extinction coefficient

The extinction coefficients for DNA and RNA are 50 and 40 respectively. The purity of the nucleic acid samples was calculated by the "260 to 280 ratio" (A_{260}/A_{280}). RNA has an A_{260}/A_{280} of 2.0 ± 0.15, and DNA has an A_{260}/A_{280} of 1.8 ± 0.15. Variations outside these ranges indicated the presence of contaminants, which had to be removed.

2.2.1.10 DNA sequencing and analysis

The DNA to be sequenced (~250ng) was mixed with 10pmol of primer in 6µl ddH_2O and sent to the University of Bath DNA Autosequencing Service. The sequencing primers used are detailed in the results chapters and nucleotide sequences for all the primers can be found in appendix A. Sequence data was analysed using the GCG (Genetic Computer Group, Wisconsin, USA) suite of programs and Multalin (Corpet, 1988).

2.2.2 Cloning of *D. immitis* GluCl subunit cDNAs

2.2.2.1 Total RNA extraction from *D. immitis*

The following procedure was conducted in a ducted fume cupboard due to the antigenic nature of *D. immitis* and the use of phenol and chloroform during the extraction. All

materials used were treated with 1% (v/v) hydrogen peroxide and autoclaved where possible to ensure they were RNase free. Solutions were made with DEPC-treated ddH_2O to ensure that they were also RNase free.

Approximately 0.3-0.5g of frozen adult female *D. immitis* was ground into a fine powder under liquid nitrogen using a pestle and mortar. The powder was scraped into a homogeniser and TriPure® Isolation Reagent (a monophasic solution of phenol and guanidine thiocyanate; Boehringer Mannheim, Lewes, U.K.) was added (1ml for every 0.1g of material). Following homogenisation (10 repeats) the mixture was left to stand at room temperature for 1-5 minutes to ensure the complete dissociation of the nucleoprotein complexes. The mixture was transferred to a 15ml Corex tube and chloroform was added (0.2ml for every 1ml of TriPure® used). After capping securely, the tube was shaken vigorously for 15 seconds and left to incubate at room temperature for 2-15 minutes. The mixture was centrifuged at 12,000g for 15 minutes at 4°C in order to separate the solution into 3 phases. The RNA containing upper aqueous phase was removed, with care taken not to remove any of the interface, and placed into another 15ml Corex tube. Isopropanol was added (0.5ml for every 1ml of TriPure® used) to precipitate the RNA, and the tube was mixed by 10 inversions and left to incubate on ice for 5-10 minutes. The RNA was recovered by centrifugation at 12,000g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 75% (v/v) ethanol (1ml for every 1ml of TriPure® used) by vortexing. Following centrifugation at 7,500g for 5 minutes at 4°C, the ethanol supernatant was removed and the pellet was allowed to air-dry for 5-10 minutes. The RNA was re-suspended in 250µl of 0.5% (w/v) SDS (made with DEPC-treated water). A sample of the RNA (2µl) was diluted in 100µl of DEPC-treated water in order to determine its concentration and purity using the spectrophotometer (section 2.2.1.9). The RNA solution was then adjusted to a concentration of 1µg/µl by addition of further 0.5% (w/v) SDS and aliquoted into 75µl samples that were stored at -70°C.

2.2.2.2 Isolation of mRNA from total RNA

The mRNA component of the total RNA was isolated using the Dynabeads® mRNA Purification Kit (Dynal, Wirral, U.K.). The following procedure was conducted under RNase-free conditions using RNase-free materials.

Buffer	Composition
Binding Buffer	20mM Tris-HCl pH 7.5, 1M LiCl, 2mM EDTA.
Washing Buffer B	10mM Tris-HCl pH 7.5, 0.15M LiCl, 1mM EDTA.

To a thawed aliquot of the *D. immitis* total RNA (75µl at 1µg/µl) 25µl of DEPC-treated ddH_2O was added. The sample was heated to 65°C for 2 minutes in order to disrupt RNA secondary structures. Meanwhile, 200µl (1mg) of the re-suspended Dynabeads Oligo $(dT)_{25}$ was removed from the stock solution and placed into a 1.5ml RNase-free tube. The tube was placed on the Dynal MPC (magnetic holder) and after 30 seconds the supernatant was removed. The tube was removed from the magnet and the Dynabeads Oligo $(dT)_{25}$ were washed by re-suspending them in 100µl of Binding Buffer. Once again the tube was placed on the magnet, after 30 seconds the supernatant was removed and the beads were then re-suspended in 100µl Binding Buffer. The total RNA was added to the Dynabeads Oligo $(dT)_{25}$ and was mixed thoroughly by rotating on a roller for 3-5 minutes at room temperature. This enabled the mRNA to anneal to the Dynabeads Oligo $(dT)_{25}$. The tube was subsequently placed on to the magnet to separate the mRNA-bound Dynabeads Oligo $(dT)_{25}$ from the other RNA components of the solution. The supernatant was removed after 30 seconds. The tube was removed and the Dynabeads Oligo $(dT)_{25}$ were washed twice with 200µl Washing Buffer B using the magnet. Care was taken to completely remove all the supernatant between each washing step. The mRNA was eluted by re-suspending the Dynabeads Oligo $(dT)_{25}$ in 10µl of the provided 10mM Tris-HCl (pH 7.5) and incubating at 65°C for 2 minutes. The tube was immediately placed on the magnet and the eluted mRNA was transferred to a new RNase-free tube. The mRNA makes up approximately 1% of the total RNA, therefore from 75µg of total RNA about 750ng should have been isolated, although some was probably lost during the procedure. However, this was an ample amount for cDNA synthesis.

2.2.2.3 First strand cDNA synthesis

First strand cDNA synthesis was performed using the SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase (Boehringer Mannheim, Lewes, U.K.). An oligo $(dT)_{12-18}$-RiRo primer (1µl of 1µg/µl stock) was added to the 10µl of eluted mRNA and 1µl DEPC-water in a nuclease-free microfuge tube. The mixture was heated at 70°C for 10 minutes and then quickly chilled on ice. To the mixture was added 4µl of 5X First Strand Buffer, 2µl of 0.1M DTT and 1µl of 10mM dNTP mix (10mM each of dATP, dGTP, dCTP and dTTP at neutral pH; 100mM stocks from Boehringer Mannheim). The contents of the tube was gently mixed and incubated at 42°C for 2 minutes at which point the SUPERSCRIPT™ II Reverse Transcriptase (1µl, 200 units) was added. Following incubation at 42°C for 50 minutes, the enzyme was inactivated by heating at 70°C for 15 minutes. The RNA was removed by addition of *E. coli* RNase H (1µl, 2 units; Promega) and incubation at 37°C for 20 minutes. This step was important to prevent complimentary RNA interfering with the cDNA in PCRs where the target was >1kb.

2.2.2.4 The polymerase chain reaction

Polymerase Chain Reactions (PCRs) were carried out in a Perkin-Elmer, Model PTC-100™ (MJ Research, Inc) thermo-cycling machine using the Expand™ High Fidelity PCR System (Boehringer Mannheim, Lewes, U.K.). This system employed an enzymatic mix of thermostable Taq and Pwo DNA polymerases. Pwo DNA polymerase has an inherent 3'-5' exonuclease proofreading ability, thus it was hoped use of this system would increase the fidelity of the PCR products. The PCRs were performed in 0.2ml thin-walled microcentrifuge tubes.

The reaction mix consisted of 34.25µl of ddH_2O, 5µl of 10X Expand High Fidelity DNA polymerase buffer (included 15mM $MgCl_2$), 7.5µl of 2mM dNTP mix (2mM each of dATP, dGTP, dCTP and dTTP at neutral pH) 1µl each of the specific oligonucleotide primer (20µM stocks; synthesised by MWG Biotech, Milton Keynes, UK), 1µl 10ng cDNA template, and 0.75µl Expand High Fidelity polymerase enzyme mix (2.6 units). The enzyme mix was usually added to the reaction mix following a 'hot-start' to the PCR. The basic PCRs used a typical 3-step program consisting of a hot start at 94°C for 2 minutes

followed by 40 cycles of 94°C for 30 seconds, 'x'°C (annealing temperature for specific primers) for 30 seconds and 72°C for 1 minute. The final extension step was 72° for 7 minutes.

Selected PCRs used a Touchdown approach. These PCRs essentially used a 3-step program, however over the first ten cycles the annealing temperature was continually lowered each time by typically 0.5/1°C to enhance the specificity of the reaction. The remaining cycles were conducted at the lowest annealing temperature. Details of each PCR, i.e. program type (conventional 3-step or Touchdown PCR), annealing temperatures and primers, can be found in the appropriate figure legends. PCR products were analysed by gel electrophoresis and products of interest were gel purified as previously described in sections 2.2.1.1 and 2.2.1.2 respectively.

2.2.2.5 Cloning of blunt-ended PCR products

Unless otherwise stated PCR products were cloned using the Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen, Groningen, Holland). This system allowed the blunt-ended PCR products, generated by the Expand™ High Fidelity PCR System, to be efficiently ligated into the plasmid vector pCR®-Blunt II-TOPO in a 5 minute reaction at room temperature. The pCR®-Blunt II-TOPO system allowed direct selection of positive recombinants via disruption of the lethal *E. coli* gene, *ccdB*, during a successful ligation. The ligation reaction mix comprised of 0.5 to 4µl of the purified PCR product (10-20ng/µl), sterile H_2O to 4µl, and the pCR®-Blunt II-TOPO vector (1µl). The ligation reaction was gently mixed and incubated for 5 minutes at room temperature. After 5 minutes 1µl of the 6x TOPO™ Cloning Stop Solution (0.3M NaCl, 0.06M $MgCl_2$) was added. The ligation reaction was mixed for 10 seconds at room temperature and then placed on ice. The addition of the Stop Solution created conditions that favour dissociation of the topoisomerase from the DNA and increased the yield of transformants. For each transformation one vial of TOP10 One Shot™ competent cells was thawed on ice. A sample of the ligation reaction (2µl) was then added. The transformation then proceeded as outlined in section 2.2.1.6, however kanamycin (50µg/ml final concentration) was used instead of ampicilin as the selective antibiotic contained within the LB-agar plates. Minipreps of the transformants were

typically analysed by digestion with EcoRI, as sites for this endonuclease were located on both sides of the multiple cloning site of pCR®-Blunt II-TOPO.

2.2.3 Expression of GluCl cDNAs in mammalian cell-lines

2.2.3.1 Maintenance of COS-7 and HEK-293T cell-lines

2.2.3.1.1 Routine cell culturing

COS-7 and HEK-293T cell-lines were routinely cultured in 75cm^2 vented flasks containing 12ml of supplemented DMEM (Dulbecco's Eagles Media supplemented with 10% (v/v) FBS, 2mM L-glutamine, and 100,000units penicillin/100mg streptomycin). These were incubated at 37°C with 5% CO_2. At approximately 85% confluence cells were harvested with 0.4ml 10x trypsin-EDTA in 4ml 1x PBS. Cells were routinely split 1:8 for confluency after 3-4 days.

2.2.3.1.2 Preparation of cells for storage in liquid nitrogen

Approximately 80% confluent cells were harvested into 10ml DMEM and centrifuged in sterile Sterilin 20ml tubes at 1,500rpm for 5 minutes. The pelleted cells were re-suspended in 1ml FBS containing 10% (v/v) DMSO and transferred to cryotubes where they were cooled slowly for 24 hours in liquid nitrogen fumes before being transferred to liquid nitrogen for storage.

2.2.3.1.3 Re-establishment of frozen cells

Frozen aliquots of cells were rapidly thawed in a 37°C water bath and added to 10ml of DMEM. Following centrifugation at 1,500rpm for 5 minutes, the supernatant was removed and the cell pellet was re-suspended in 15ml DMEM. After transferal to a 75cm^2 vented flask the cells were maintained as described in section 2.2.3.1.1.

2.2.3.2 Transient expression procedure

2.2.3.2.1 Preparation of COS-7 and HEK-293T cell-lines for transfection

Prior to transfection the cells were split 1:10 and then 1ml of the culture was further diluted in 12-15ml (depending on level of confluency of original cells) of DMEM. The diluted culture was then added drop-wise (typically 3-5) on to coverslips that had been placed in 6-well plates. After 1 hours incubation, sufficient time to enable cell adherence to the coverslip, 2mls of DMEM was added to each well and the plates were incubated overnight at 37°C with 5% CO_2. Following this period the cells had usually reached the desired confluency (40-50% COS-7 cells; 20-30% HEK-293T cells) for the transfection procedure to be undertaken. If the cells had not reached this level of confluency they were left to grow until it was achieved before starting the transfections. Although the COS-7 cells strongly adhered to the coverslips the HEK-293T cells had a tendency to float off during application of the medium. Therefore before plating these cells the coverslips were first treated with PEI (described below).

2.2.3.2.2 Treatment of coverslips with polyethylenimine (PEI)

PEI (1g) was dissolved in 10ml of ddH_20 and filter sterilised (using a 0.22μM filter). A 1:100 dilution was prepared from the stock with sterile 0.1M Borax and 2ml was added to each coverslip in a 6 well plate. These plates were incubated for at least 2 hours at 37°C before the PEI solution was removed by aspiration. The plates were subsequently washed 3 times with PBS before the HEK-293T cells were plated as described above.

2.2.3.2.3 Calcium phosphate-mediated transfection

3-4 hours prior to transfection the medium was changed, during which period the transfection mixture was prepared. To one 1.5ml tube was added 12μl 2M $CaCl_2$, 1-10μg DNA (depending on sample) and sterile ddH_2O up to 100μl total volume. To a second tube was added 100μl 2x HEPES Buffered Saline (HBS). The DNA mixture was then added portion-wise (20μl) to the HBS, the tube being shaken between additions to increase aeration of the mixture and consequently precipitation formation. Once the DNA had been completely added, the mixture was incubated at room temperature for 30 minutes after which time the newly-form precipitates were added, drop-wise, to the appropriate well of

cells. The cells were then left to incubate overnight at 37°C with 5% CO_2. The following day the cells were washed twice with PBS in order to remove the precipitate and 2ml of fresh medium was added to the cells. The cells were then incubated for a further 24-48 hours at 37°C with 5% CO_2. Variations upon the method described, used during the optimisation process (see chapter 4), included incubating the cells in the DNA-calcium phosphate mixture for only 5-6 hours as suggested in some protocols. In additions cells on occasion were subjected to a "glycerol shock", following removal of the transfection mixture, to increase uptake of the DNA. This involved incubating the cells in 15% (v/v) glycerol (in HBS) for 2 minutes before washing with PBS and re-incubating the cells in medium.

2.2.3.3 Immunocytochemistry

After 24-48 hours incubation the cells were washed three times with 2ml of PBS. The cells were fixed by incubation in 2ml 4% (w/v) paraformaldehyde (in PBS) for 20 minutes at room temperature. Following 3 washes with 2ml PBS, the cells were permeabilized by incubation with 2ml 0.1% (v/v) Triton X-100 (in PBS) at room temperature for 5 minutes. After a further 2 washes with PBS the samples were blocked for 30 minutes with 2ml of 10% (v/v) Horse serum (in PBS). The coverslips were then removed, inverted and placed on to 70µl of the primary antibody solution for 1 hour. The primary antibodies used were either anti-Flag M2® mouse IgG1 monoclonal antibody at a dilution of 1 in 1500 in 10% (v/v) horse serum or anti-*c-myc* mouse IgG1 monoclonal antibody at a dilution of 1 in 200 in 10% (v/v) horse serum. After incubation with the primary antibody the coverslips were carefully removed and placed back, cells up, into the original 6 well plates. The samples were washed four times in PBS, each wash lasting five minutes. After the final wash the coverslips were removed, inverted and placed on to 70µl of the secondary antibody solution for 20 minutes. The secondary antibody used for both primaries was the Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG (1 in 200 dilution in 10% (v/v) horse serum). The coverslips during this step and subsequent steps were covered as much as possible to reduce the exposure of the secondary antibody to the light. Once again following incubation, the coverslips were removed, placed cells up, and washed four times with PBS, each wash lasting for 5 minutes. Finally the coverslips were removed, inverted, and mounted on to coverslides using 30µl of the mounting medium Mowiol (obtained from

V. Portillo; prepared as described by Harlow & Lane, 1988) on to a coverslide. The slides were left to dry overnight at room temperature and then placed at 4°C until viewed. Slides for Green Fluorescent Protein (GFP), the control used to optimise the transfection reaction, were mounted following the fixation step. Protein expression was investigated by viewing the slides with a Zeiss LSM 510 inverted Axiovert 100M confocal microscope using a fluorescein filter set.

2.2.4 Functional expression of GluCls in *Xenopus* oocytes

2.2.4.1 Synthesis of capped GluCl RNA for injection

2.2.4.1.1 Preparation of template DNA

Before *in vitro* transcription of cRNA could be performed the template DNA had to be prepared. The GluCl subunit cDNAs of interest were sub-cloned, using methods described in section 2.2.1, into plasmids containing T7 promoters upstream of their start codons. Details of the plasmids used for each subunit cDNA can be found in the results chapter. The recombinant plasmids (~12ug) containing full-length cDNAs for the GluCl subunits were linearised at the 3' end by restriction digestions (section 2.2.1.3). The resulting template DNA was purified by phenol:chloroform extraction and ethanol precipitation. Three volumes of phenol:chloroform (Tris-saturated) were added to the sample which was vortexed vigorously and briefly centrifuged in order to collect the upper DNA-containing phase. A second extraction was carried out on the upper phase, in a similar manner, with the addition of an equal volume of chloroform. To precipitate the DNA sodium acetate (30µl; pH5.2) and absolute ethanol (720µl) were added to the sample, which was chilled for at least 15 minutes at -20°C. The DNA was recovered by centrifugation at 20,000g for 15 minutes at 4°C after which the supernatant was removed and the pellet was re-suspended in 12µl of DEPC-ddH_2O. A sample of the DNA (~2µg) was run on an agarose gel (section 2.2.1.1) to ensure complete linearisation of the recombinant plasmid.

2.2.4.1.2 Transcription of capped RNA

Capped RNA was generated using the T7 RNA polymerase mMESSAGE mMACHINE™ Kit (Ambion, Huntingdon, UK). The capped RNA transcription reaction comprised 10µl 2x NTP/Cap, 2µl 10x reaction buffer, 1µg linear template DNA, 2µl enzyme mix and nuclease-free water to 20µl. The reaction contents was gently mixed and incubated at 37°C for 1-2 hours. To ensure the template DNA did not interfere with subsequent procedures DNase I (included in the kit) was added (1µl) and the sample was incubated for a further 15 minutes. Recovery of the capped RNA was achieved by phenol:chloroform extraction and isopropanol precipitation. Nuclease-free water (115µl) and ammonium acetate stop solution (15µl), both included in the kit, were added to the RNA reaction and mixed thoroughly. The RNA was first extracted by the addition of 1 volume of phenol:chloroform (acidic), which was vortexed vigorously and briefly centrifuged in order to collect the upper RNA-containing phase. A second extraction was carried out on the upper phase, in a similar manner, with the addition of an equal volume of chloroform. To precipitate the RNA isopropanol was added and the mixture was chilled for at least 15 minutes at -20°C. The RNA was recovered by centrifugation at 20,000g for 15 minutes at 4°C after which the supernatant was removed and the pellet was re-suspended in 10µl nuclease-free water, supplied with the kit. The RNA concentration was determined by spectrophotometry (section 2.2.1.9) and adjusted to 1µg/µl if the purity given by the A_{260}/A_{280} fell within the acceptable limits (2.0 ± 0.15). Aliquots of 2µl were then stored at -70°C.

2.2.4.1.3 Analysis of capped RNA by denaturing gel electrophoresis

The quality of the RNA also had to be examined by denaturing gel electrophoresis to ensure only one band of the correct size had been transcribed. Prior to preparing the gel the electrophoresis apparatus (gel tank and cast) was soaked in 1% (v/v) hydrogen peroxide for at least 30 minutes to ensure the removal of contaminant RNases. The hydrogen peroxide was removed by rinsing the apparatus with DEPC-ddH_2O. To prepare the denaturing gel initially 0.75g agarose was dissolved in 36ml ddH_2O. Once the molten agarose had cooled to approximately 55°C, 5ml of 10x MOPS and 9ml formaldehyde (37% solution) were added and gently mixed by swirling. Subsequently, the mixture was poured into the gel apparatus. Once set, the comb was removed and the gel was submerged into a tank of 1x MOPS. Before loading, the RNA samples had to be de-natured. The denaturation reaction

was comprised of 2-5μg RNA, 2ul 10x MOPS, 4μl formaldehyde solution, 1μl ethidium bromide (200μg/ml stock concentration) and formamide solution up to 18μl, which were incubated for 5 minutes at 85°C. Following cooling 2μl of 10x formaldehyde loading buffer were added and the samples were loaded on to the gel. The samples were electrophoresed at 100V until the marker dye had sufficiently migrated and then visualised under UV light. An RNA Ladder from New England Biolabs (Hitchen, Hertfordshire) was used to determine the sizes of the cRNA samples. Before loading, the RNA ladder (5μl) also had to be denatured in the way described above.

2.2.4.2 *Xenopus* oocyte preparation and injection

Ovarian tissue was surgically removed from sexually mature female *Xenopus laevis* following euthanasia with an overdose of 0.05% (w/v) benzocaine. The isolated ovarian lobes, maintained in OR-Mg, were dissected into smaller clumps and placed into OR-Mg containing 1.5mg/ml collagenase (type 1A) for 1.5-2 hours until approximately 50% of the oocytes had no follicle surrounding them. After washing 3 times with OR-Mg, stage V and VI oocytes were selected and incubated overnight in ND96 at 18°C on a gently rotating tabletop shaker.

Injection needles were pulled from 3.5 Drummond glass capillary tubing (Clark Electromedical, U.K.) on a stationary coil Narishige microelectrode puller (Optical Instruments Services Ltd., Croyden, U.K.) to produce a tip of approximately 1μM, which was then gently broken to give a tip with a slightly larger diameter. Needles were back filled with sterile mineral oil and attached to the plunger of a Drummond "Nanoject" microinjector (Broomhall, PA, U.S.A.). With the aid of a binocular microscope oocytes were injected cytoplasmically with either 50ng (1ng/nl) of the selected GluCl RNA or 50nl of nuclease-free H_2O (injection control group). An uninjected control group was also established. The oocytes were left to incubate, with daily ND96 buffer changes, for 48-96 hours before recording.

2.2.4.3 Two-electrode voltage clamp recordings

Microelectrodes were pulled from borosilicate glass capillaries on a microelectrode puller to produce a resistance, when filled with 3M KCl, of between 0.5 and 5 MΩ. Each oocyte

was placed in a Perspex-recording chamber, which was continuously perfused with ND98 (from a gravity fed system at a rate of 5ml/minute). The oocyte was subsequently impaled by the microelectrodes. Electrophysiological recordings were performed using a two-electrode voltage clamp amplifier (GeneClamp 500B, Axon Instruments) on oocytes with a resting membrane potential of greater than –25mV and maintained at a holding potential of E_h= –80mV. In response to applied drugs, e.g. glutamate or ivermectin, the change in current (or lack of) across the membrane of the oocyte required to keep the oocyte clamped at –80mV was measured on an 800Mhz Pentium III PC using the data acquisition software pClamp8 (Axon Instruments). Data were digitised using a Digidata 1322A (Axon Instruments).

2.2.4.4 Data analysis

Data were analysed using the program Graphpad Prism version 3 (Graphpad Software, San Diego, U.S.A.) and presented as the mean ± the standard error of the mean. Hill coefficients were calculated using the following equation:

$$I = I_{min} + (I_{max} - I_{min})/(1 + 10^{[(logEC50 - [ag])H]})$$

This equation was used to fit a sigmoid curve of variable slope to the normalised data. I_{max} and I_{min} represent the (percentage) maximal and minimal currents induced by a particular agonist. The EC_{50} is the concentration of agonist necessary to elicit half the maximum response, H is the (Hill) slope coefficient, and [ag] is the log of the agonist concentration.

2.2.4.5 Ligand application protocol

All ligands were dissolved in ND98. Saline insoluble IVM was dissolved in absolute ethanol (<1% v/v final concentration in saline); this solvent concentration was shown to have no undesirable effects either on drug-induced responses or on uninjected oocyte membranes when cells were clamped at E_h = –80mV. Only those oocytes, which gave stable responses to at least 2 applications of 1mM L-glutamate, were used.

L-glutamate-dose response curves were generated by initially challenging the expressed channels with 1mM glutamate and then two increasing concentrations of the ligand. Five-

minute intervals were observed between each application to prevent desensitisation. Every third application 1mM L-glutamate was applied to check for "run-down" of the electrophysiological responses. Dose response protocols were not conducted for IVM but rather singular concentrations were applied to oocytes in order to obtain qualitative responses. A separate perfusion system and recording chamber was used for IVM experiments, which was washed thoroughly with absolute ethanol between applications due to the highly hydrophobic nature of the compound.

2.2.5 Miscellaneous *C. elegans* methods

2.2.5.1 Routine culturing of *C. elegans*

The Bristol N2 strain of *C. elegans* was routinely maintained on NGM agar plates seeded with a lawn of OP50, the bacterial food source. Plates were incubated at 20°C and sub-cultured every 4-7 days by transferring worms to a new plate using a wire loop. This was conducted using standard microbiological sterile precautions at the bench; the worms were visualised under a dissecting microscope (Zeiss, Germany).

2.2.5.2 Total RNA extraction from *C. elegans*

C. elegans total RNA was extracted using a similar methodology to that used for *D. immitis*, the major difference being the initial homogenisation step, which is described here. Further steps past that point are described in section 2.2.2.1.

During routine sub-culturing *C. elegans* were transferred to three large NGM plates (9cm diameter). These plates were incubated for a suitable period until they were considered to be "just clearing", i.e. the density of worms on the plate was at or approaching the maximum. M9 buffer was then used to wash the worms from the plates into 15ml Falcon tubes, which were subsequently centrifuged at 150g for 1 minute. Approximately 200µl of "packed" worms were transferred to microfuge tubes, which contained 0.5ml of glass beads (425-600 microns, acid washed, Sigma). TriPure® (0.8ml) was added and the worms were homogenised by vortexing the tubes for 1 minute. Following vortexing, the homogenate was centrifuged briefly. The supernatant was subsequently drawn off the beads and placed

into clean 1.5ml microcentrifuge tubes. The beads were then rinsed with a further 200µl TriPure®, vortexed briefly and centrifuged as before. Once again the supernatant was removed and combined with that removed previously. From this point the method used for *C. elegans* was similar to that used for *D. immitis*, only differing in scale, meaning the process was carried out in microfuge rather than Corex tubes. The reader is referred, as stated, to section 2.2.2.1 for the chloroform wash onwards.

2.2.5.3 RNA-mediated interference protocol

NGM plates (6cm diamater) were prepared that contained 1mM IPTG and 50µg/ml ampicilin. Meanwhile, the HT115(DE5) bacteria containing the L4440 plasmid constructs (*unc-22*, gene of interest and empty) were grown overnight. The following day selected plates were seeded with one of the three bacterial cultures (~100µl spread as a lawn) and left overnight at room temperature. *C. elegans*, of different larval stages, were then transferred on to the plates (approximately 20 on each), which were subsequently incubated at 20°C. Each day the worms on all three plasmid constructs were monitored for the development of any gross phenotypes. The *unc-22* phenotype, which consisted of affected worms exhibiting strong twitching, developed after approximately 48 hours and was therefore used as the positive control for the procedure.

Chapter 3

The Cloning of DiGluClα3A and α3B Subunit cDNAs

3.1 Introduction

The first GluCl subunit cDNAs (GluClα1 and GluClβ) were isolated from the model nematode *C. elegans* (Cully *et al.*, 1994). Alignment of the clones' sequences with nucleotide and protein databases revealed their high homology to both glycine and $GABA_A$ receptor subunits. This placed the GluCls firmly in the chloride channel "branch" of the LGIC superfamily, of which the muscle nAChR is considered the prototype member. There are a number of characteristic motifs exhibited by members of this superfamily, GluCl subunits included (Ortells & Lunt, 1995). A cleaved signal peptide is followed by a large N-terminal extracellular domain, which contains potential *N*-linked glycosylation sites, and a pair of cysteine residues that form a disulphide bridge or loop. Indeed, members of this superfamily are sometimes referred to as the "cys-loop" receptors to distinguish them for other types of LGICs, such as the cationic glutamate receptors. Common to only the GluCl and glycine subunits is the presence of a second pair of disulphide-linked cysteine residues, downstream of the first (Vassilatis *et al.*, 1997b). These are distinct from the vicinal cysteine residues present in α-type nAChR subunits. The C-terminal contains four hydrophobic transmembrane domains (TM1-TM4), between the third and fourth of which exists a long intracellular loop that contains consensus sequences for phosphorylation by protein kinases (Ortells & Lunt, 1995). As inferred from studies principally on nAChRs, mature LGICs are presumed to be homo- or heteropentamers, depending on the constituent subunits, the TM2 of each polypeptide lining the ion pore (Unwin, 1993).

In *C. elegans* six genes encode for eight GluCl subunits, the *avr-15* and *avr-14* genes being alternatively spliced to produce two subunits each (Cully *et al.*, 1994; Dent *et al.*, 1997; Laughton *et al.*, 1997a; Vassilatis *et al.*, 1997a; Horoszok *et al.*, 2001). Analysis of the completed *C. elegans* genome indicates that there are no other likely candidate GluCl genes (The *C. elegans* Sequencing Consortium, 1998) In *H. contortus*, the parasitic nematode in which GluCls have been most extensively studied, three genes have so far been described. Two of these genes, *glc-2* (encoding the HcGluClβ subunit) and *avr-14*, are clearly orthologous to those found in *C. elegans* (Delany *et al.*, 1998; Jagannathan *et al.*, 1999). The third gene, encoding a HcGluClα subunit (Forrester *et al.*, 1999), does not appear to be orthologous to any found in *C. elegans*, indicating that there are differences in the genetics of the GluCls between even quite closely related nematode species: *H. contortus* and *C.*

elegans are in the same clade of the phylogenetic tree proposed by Blaxter *et al* (1998a). Partial GluCl cDNAs, all corresponding to *avr-14*, have also been isolated from *D. immitis*, the closely related filarial species *O. volvulus* (Cully *et al.*, 1996a), and *A. suum* (Jagannathan *et al.*, 1999).

The alternative splicing of the *avr-14* gene (illustrated in figure 3.1), conserved between *C. elegans* and *H. contortus*, produces two mRNA that encode for the GluClα3A and α3B subunits. Although both subunits share a common N-terminal extracellular domain, which presumably includes the glutamate binding site, they have differing C-terminal channel forming domains. The coding region for the GluClα3B subunit C-terminal is located within a large 3'-untranslated region (UTR) of the GluClα3A mRNA (Laughton *et al.*, 1997a; Jagannathan *et al.*, 1999).

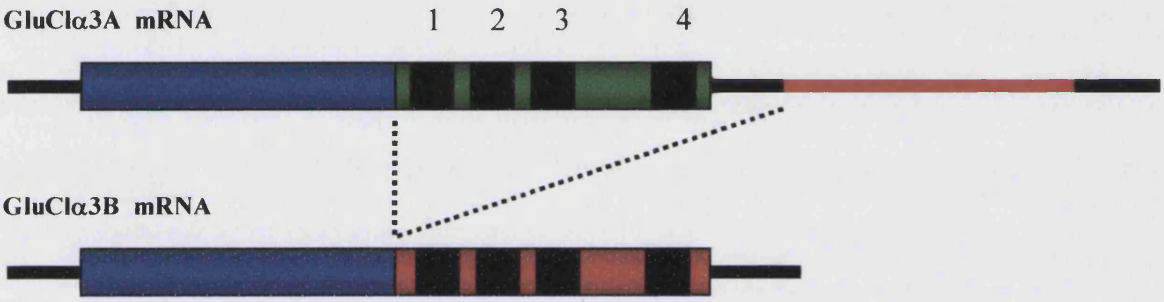


Figure 3.1. The alternative splicing of the *avr-14* gene in *C. elegans* and *H. contortus*. Coding regions are shown as boxes and non-coding regions are shown as lines. The GluClα3 mRNAs encode for common N terminal glutamate-binding domains (blue boxes) attached to differing C terminals (red & green boxes), which contain the TM regions (numbered black boxes). Within the large 3'-UTR of the GluClα3A mRNA is the alternative C-terminal coding region (red line), which is spliced (indicated by the broken lines) to the common N-terminal coding region to generate the GluClα3B mRNA. The GluClα3A and α3B mRNAs are ~2.1 and ~1.5kb in size respectively (Laughton *et al.*, 1997a; Jagannathan *et al.*, 1999;).

The initial objective of the project was to amplify a full-length *D. immitis avr-14* cDNA corresponding to the partial sequence (termed GluClX in GenBank) that had already been cloned. A homology search using the partial sequence indicated the full-length cDNA would most likely encode a polypeptide orthologous to the GluClα3A subunits of *C. elegans* and *H. contortus*. It was further hypothesised that a GluClα3B subunit cDNA could

also be amplified from *D. immitis* given that the partial *avr-14* cDNAs from the closely related species *O. volvulus* and *A. suum*, when translated, exhibited greater identity to this alternatively spliced form. Using degenerate PCR it was hoped that further *D. immitis* GluCl subunit cDNAs could also be amplified.

The cloning strategy heavily involved the PCR strategy, RACE (Rapid amplification of cDNA ends). Using this method, oligonucleotide primers external to the sequence of interest are used in tandem with internal primers to a known part of the sequence to amplify the unknown 5' and 3' terminals (Frohman *et al.*, 1988). In order to deduce the full-length coding region the RACE PCRs were designed to amplify overlapping 5' and 3' cDNAs. 5' RACE in nematodes is facilitated by the spliced leader (SL) 1 sequence, which was used during the cloning process as the external sense primer to amplify the unknown 5' terminal. SL1 is a highly conserved, 22-nucleotide sequence, which is *trans*-spliced from a small (~100-nucleotide) SL RNA on to the 5' terminals of a large proportion of pre-mRNA in nematodes. Despite its highly conserved nature the exact function of the SL1 sequence remains unclear (Nilsen, 1993, Nilsen, 1995; Blumenthal, 1995). The external, anti-sense primer (RoRi) used in 3' RACE was designed to a 3' adaptor site, RiRo, incorporated during cDNA synthesis from isolated *D. immitis* mRNA.

3.2 Results and Discussion 1:

Amplification of DiGluClα3 cDNAs

3.2.1 Preparation of *D. immitis* cDNA

The initial step in the preparation of *D. immitis* cDNA, the template for subsequent PCRs, was the extraction of total RNA. Adult female microfilaria-bearing worms were selected for the extraction procedure. This increased the chances of amplifying rare transcripts, such as LGIC mRNAs, as both adult and larval mRNA pools were isolated. Figure 3.2 shows a sample (~2μg) of successfully extracted total RNA, analysed by conventional agarose gel electrophoresis. Two ribosomal bands and a smaller band, relating to the tRNAs were typically visualised.

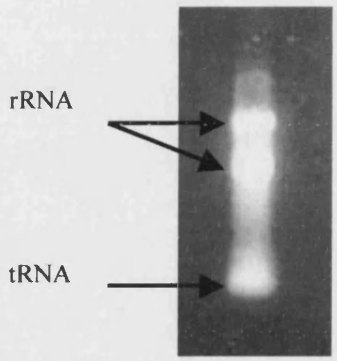


Figure 3.2. *D. immitis* total RNA. A 2μg sample was electrophoresed on a 1.5% agarose gel. The arrows indicate the rRNA and tRNA components.

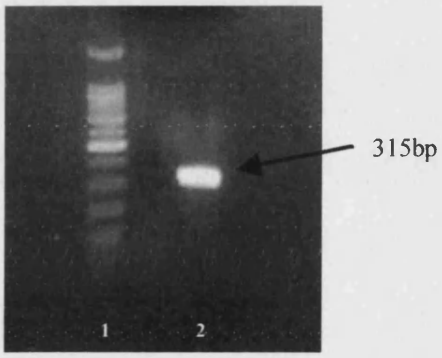


Figure 3.3. Amplification of a partial *D. immitis avr-14* cDNA. Lane 1: 100bp DNA ladder; lane 2: PCR product, amplified using a 3-step PCR program (annealing temperature of 52°C, extension time of 30 seconds) and primers 5Di2/3P1 & 3Di2/3P1.

The cDNA was synthesised by reverse transcription from the poly(A)$^+$ selected mRNA component of the total RNA. A control PCR to assess the quality of the cDNA was successfully performed, using oligonucleotide primers (5Di2/3P1 and 3Di2/3P1) designed to amplify a 315bp region of the partial *D. immitis avr-14* cDNA (figure 3.3). The PCR product was subsequently cloned into pCR®-Blunt II-TOPO, recombinant clones being identified by restriction digest with EcoRI. Sequencing of one such clone, using the vector specific M13 primers supplied with the cloning kit, confirmed the desired region had been

amplified. The nucleotide sequences of these and all other primers used in this study can be found in appendix A.

3.2.2 Amplification of the DiGluClα3A cDNA

5' and 3' RACE, graphically illustrated in figure 3.4, was used to obtain overlapping cDNA clones from which a DiGluClα3A nucleotide sequence could be deduced. Using the anti-sense primer 3Di2/3P1 in conjunction with a sense primer to the SL1 sequence, the 5' RACE PCR amplified two cDNAs of 600 and 750bp (figure 3.5A). These products were subsequently cloned into pCR®-Blunt II-TOPO. As before, recombinant clones were identified by restriction digest with EcoRI and a selected clone was sent for sequencing using the M13 primers. Both cDNAs exhibited identical sequence, over the predicted region, with the previously cloned partial *avr-14* cDNA indicating they were all amplified from the same transcript. The 750bp cDNA exhibited an open reading frame with a putative start codon located 15bp downstream of the SL1 sequence. As predicted, translation of the open reading frame revealed a partial amino acid sequence that exhibited highest identity to the GluClα3A subunits (~80%). Identity ranged from 70-80% with GluClα3B subunits. The 600bp cDNA appeared to the result of mis-annealing of the SL1 primer downstream of the actual SL1 site, otherwise the sequence was identical to that of the 750bp product.

Figure 3.4. Schematic representation of the RACE strategy employed to amplify the DiGluClα3A subunit cDNA. The boxed regions represent the DiGluClα3A mRNA sequence; yellow: SL1 sequence; grey regions: unknown mRNA sequence; red region: partial, known *avr-14* sequence; orange poly(A) tail; green region: 3' RiRo adaptor site. The opposing black arrows indicate the region that was to be amplified during 5' RACE and the primers that were to be used. Conversely, the opposing blue arrows indicate the region that was to be amplified during 3' RACE and the primers that were to be used.

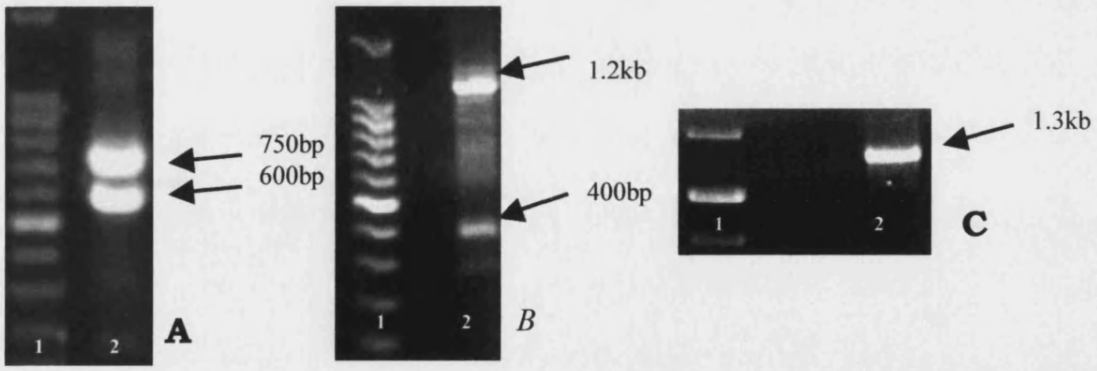


Figure 3.5. Amplification of partial and full-length GluClα3A cDNAs. Lane 1: 100bp DNA ladder (A & B); 1kb DNA ladder (C). Lane 2: PCR products. Each PCR used a standard 3-step program. **A. 5' RACE PCR** (primers SL1 & 3Di2/3P1; annealing temperature of 55°C); **B: 3' RACE PCR** (primers 5Di2/3P1 & RoRi; annealing temperature of 52°C); **C: Full-length coding region PCR** (primers SDG2 & ASDG2; annealing temperature of 51°C).

Using the sense primer 5Di2/3P1 with the anti-sense primer to the 3' adaptor site, RoRi, the 3' RACE PCR amplified two cDNAs of approximately 1.2kb and 400bp (figure 3.5B), the larger product being cloned and sequenced as before. Analysis of the sequence revealed an open reading frame with a putative stop codon that when translated exhibited highest amino acid identity, as predicted, to the GluClα3A (80%) subunits. Again, identity to the GluClα3B alternatively spliced forms was lower, ranging from 70-80%. From the overlapping 5' 750bp and 3' 1.2kb cDNAs a full-length open reading frame (i.e. the coding region) of 1260 nucleotides was deduced. The coding region was amplified, using primers SDG2 and ASDG2 (figure 3.5C), cloned as before, and fully sequenced on both strands to ensure confidence in the nucleotide sequence. However, several nucleotide substitutions were observed between the amplified coding region cDNA and the compiled sequence. Therefore to ascertain a consensus nucleotide sequence, a second PCR of the coding region was carried out. The resulting cDNA was identical in sequence to the first coding region clone and was thus adopted as the consensus sequence for the DiGluClα3A coding region (see appendix B). The proposed 1260 nucleotide sequence therefore encodes a polypeptide of 419 amino acids (figure 3.10). Addition of the 5' and 3' UTRs, determined from the overlapping RACE PCR products, meant the full-length GluClα3A mRNA was proposed to be ~1.5kb (see appendix B). Interestingly, the proposed mRNA did not exhibit the large

3'-UTR observed for the orthologous subunit mRNAs of *C. elegans* and *H. contortus*, raising the possibility that only one GluClα3 subunit may exist in *D. immitis*. However, as outlined in the introduction, partial GluClα3B-like cDNAs had been cloned from both *O. volvulus* and *A. suum*, species more closely related to *D. immitis* than *C. elegans* and *H. contortus*. It was therefore hypothesised that *D. immitis* also possessed two GluClα3 subunits but the alternative splicing mechanism or mRNA processing in this species was somehow different from that seen in *C. elegans* or *H. contortus*.

3.2.3 Amplification of the DiGluClα3B subunit cDNA

The PCR strategy devised to amplify a DiGluClα3B subunit cDNA assumed that the alternative splicing of the *avr-14* gene was conserved between this filarial species, *C. elegans* and *H. contortus* (see figure 3.1), but the large 3'-UTR was simply not maintained in the mature DiGluClα3A mRNA. Therefore, a PCR using the sense primer (SDG2) to the DiGluClα3A coding-region in conjunction with the anti-sense primer RoRi was predicted to amplify both subunit cDNAs. In addition, both subunit cDNAs were expected to be of a

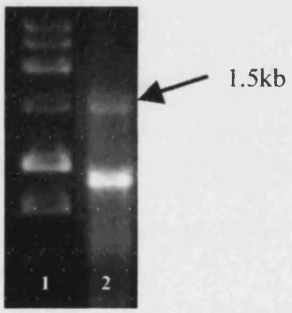


Figure 3.6. Amplification of GluClα3 subunit cDNAs. Lane 1: 1kb DNA ladder; lane 2: PCR product, amplified using a 3-step PCR protocol (annealing temperature of 49°C) with the primers SDG2 and RoRi.

similar size (~1.5kb) as they would be in *C. elegans* and *H. contortus* if the presence of the large 3'-UTR were removed. The PCR amplified a number of bands including one of 1.5kb, which was subsequently cloned into pCR®-Blunt II-TOPO (figure 3.6). As this band was predicted to be a heterogeneous mix of PCR products individual clones were screened by restriction digestion. The endonuclease EcoRI was chosen for the screening process, as there was a conserved restriction site at the centre of the GluClα3A nucleotide sequences of *C. elegans*, *H. contortus* and *D. immitis* that appeared to be absent in the GluClα3B nucleotide sequences. The expected restriction digestion pattern of the GluClα3A clones was one band at ~3kb, the linearised plasmid (pCR®-Blunt II-TOPO), and a smaller band of

750bp, the bisected insert. GluClα3B cDNAs were expected to exhibit the band at 3kb (the linearised plasmid) and a differing, although unknown, digestion pattern from that of GluClα3A cDNAs. The results of the restriction digest screen are shown in figure 3.7 with clones 3, 6, 7, 8, and 10 giving the expected digestion pattern for a GluClα3A cDNA. Systematic sequencing of the other clones indicated that clone 5, contained a potential (an intron was located at the centre of the cDNA) open reading frame of 1284 nucleotides that encoded for a GluClα3B-type subunit; homology searches revealed the predicted

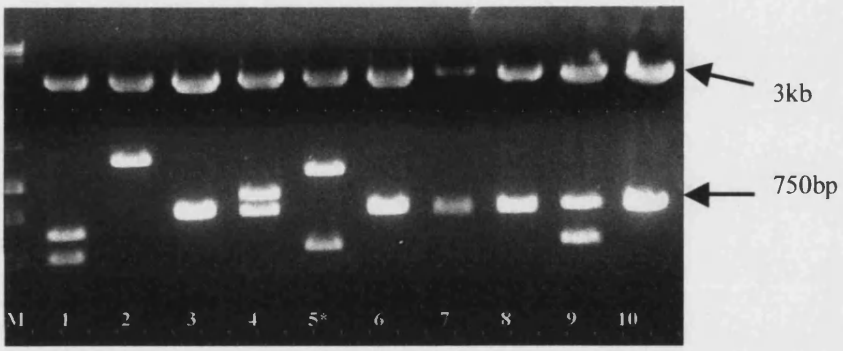
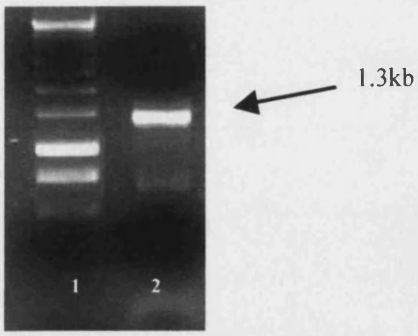


Figure 3.7. Restriction digestion screening of potential GluClα3B subunit cDNAs. Lane M: 1kb DNA ladder; lanes 1-10: individual clones screened by restriction digestion with EcoRI. The arrows indicate the expected sizes of digested GluClα3A clones. The asterisk indicates the GluClα3B clone.

polypeptide to have ≥80% and 70-80% amino acid identities with orthologous GluClα3B and GluClα3A subunits respectively. The 5' nucleotide sequence was identical to that of the DiGluClα3A coding region where as the 3' sequences of each differed, appearing to affirm the hypothesis that the alternative splicing of the *avr-14* gene was conserved across examined species. An anti-sense primer (ASDG3) was subsequently designed and used in conjunction with the sense primer SDG2 to amplify the proposed coding region of DiGluClα3B (figure 3.8). The PCR product, which was of the expected size (~1.3kb), was cloned and fully sequenced on both strands. This cDNA did not contain any introns and the sequence confirmed the predicted open reading frame. Therefore the *D. immitis* GluClα3B coding region was proposed to be 1284 nucleotides (see appendix B) making the encoded subunit 427 amino acids in length (see figure 3.10). Addition of the 5' and 3' UTRs meant the full-length DiGluClα3B mRNA was proposed to be ~1.5kb (see appendix B), similar in size to the orthologous subunit mRNAs from *C. elegans* and *H. contortus*.

Figure 3.8. Amplification of the DiGluClα3B coding region. Lane 1: 1kb DNA ladder; lane 2: PCR product. The amplification used a Touchdown PCR protocol (annealing temperature 55-50°C) with the primers SDG2 and ASDG3.



3.2.4 Sequence analysis of the DiGluClα3A and α3B subunits

As indicated in the previous sections, the amplified GluCl cDNAs from *D. immitis* encode polypeptides that are clearly orthologous to the GluClα3A and α3B subunits identified in other species of nematode, exhibiting ≥80% amino acid identity; this level of homology is comparable to that between the GluClβ subunits of *C. elegans* and *H. contortus*. It is apparent from aligning the two DiGluClα3 subunit amino acid sequences (figure 3.10) that the pattern of alternative splicing of the *avr-14* gene in this species is identical to that observed in *C. elegans* and *H. contortus*, i.e. the resulting subunits share identical N-terminal domains but have differing C-terminal domains, the splice site being located just prior to TM1. However, the final processing of the *D. immitis* GluClα3A mRNA is evidently different; the large 3'-UTR in the filarial transcript is absent (see appendix B). It appears reasonable to suggest that in *D. immitis* the 3'-UTR may be present as part of a precursor mRNA (analogous to the mature GluClα3A mRNA in *C. elegans* and *H. contortus*) and that once the "instruction" to produce a GluClα3A subunit is made, this region is cleaved, leaving the mature transcript. Indeed, this would normally be the expected scenario as the maintenance of splice sites downstream of a stop codon is normally detected by quality control mechanisms and the mRNA is degraded. The reason the 3'-UTR is maintained in *C. elegans* and *H. contortus* is unknown. Figure 3.9 graphically summarises the mRNA generated following alternative splicing of the *avr-14* genes in *C. elegans*, *H. contortus* and *D. immitis*.

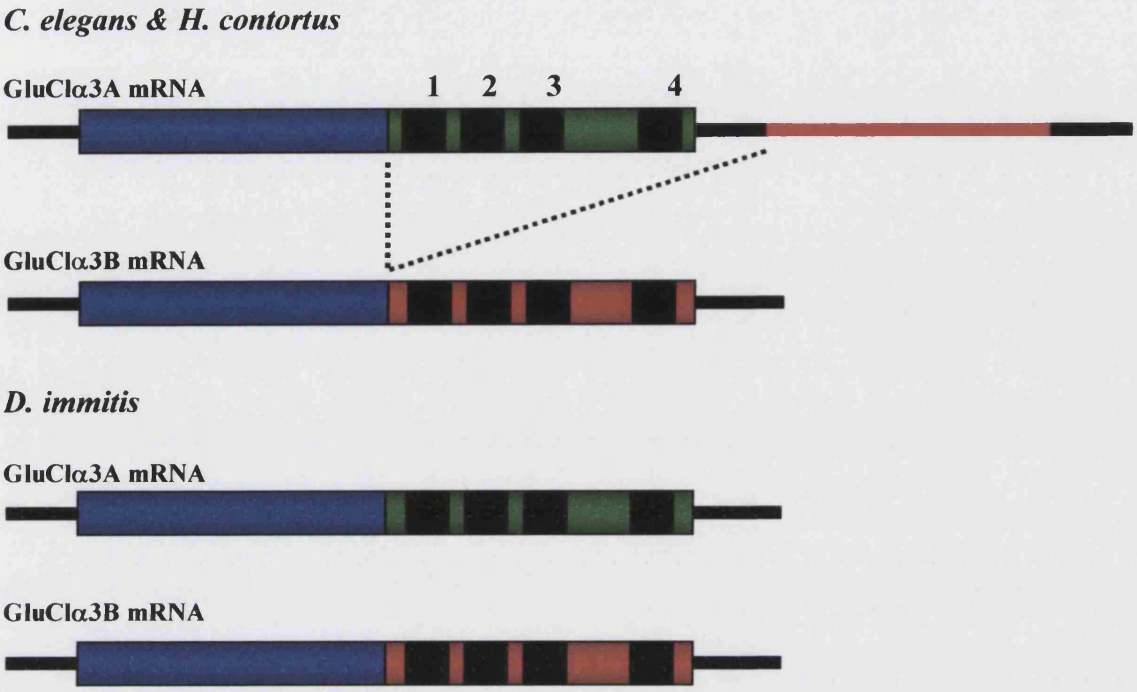


Figure 3.9. Comparison of the mRNA generated following the alternative splicing of the *avr-14* genes in *C. elegans*, *H. contortus* and *D. immitis*. Coding regions are shown as boxes and non-coding regions are shown as lines. In all three species the GluClα3 mRNAs encode common N terminal glutamate-binding domains (blue boxes) attached to differing C terminals (red & green boxes), which contain the TM regions (numbered black boxes). In *C. elegans* and *H. contortus*, within the large 3'-UTR of the GluClα3A mRNA is the alternative C-terminal coding region (red line), which is spliced (indicated by the broken lines) to the common N-terminal coding region to generate the GluClα3B mRNA. In both species the GluClα3A and α3B mRNAs are ~2.1 and ~1.5kb in size respectively (Laughton *et al.*, 1997a; Jagannathan *et al.*, 1999]. The large 3'-UTR is absent in the *D. immitis* GluClα3A mRNA leaving both subunit transcripts of a similar size (~1.5kb). Presumably the 3'-UTR is cleaved during mRNA processing in this species.

As expected, the *D. immitis* GluClα3 subunits exhibit the characteristic motifs associated with members of the LGIC superfamily. These are highlighted in figure 3.10, which shows an alignment of these subunits with other ligand-gated chloride channel (LGCC) members of the superfamily, and are discussed below. A more extensive alignment of GluCl subunit amino acid sequences can be found in appendix C. At the common N-terminal of the *D.*

immitis GluClα3 subunits is a putative signal peptide of 18 amino acid residues, as predicted by the program SignalP V1.1 (Nielsen *et al.*, 1997). As with other such polypeptides, the signal peptide would direct the N-terminal (and indeed the smaller C terminal) of these GluCl subunits into the lumen of the endoplasmic reticulum (ER) before being cleaved. It follows that correct insertion into the ER leads to the proper orientation of the receptor within the plasma membrane, i.e. the large N-terminal region will be extracellular. Within the ER the N-terminus is also exposed to the enzymes that regulate the necessary post-translational modifications, e.g. *N*-linked glycosylation and disulphide bridge formation (Green & Millar, 1995). The mature GluClα3A and α3B subunits, following cleavage of the signal peptides, would therefore be 401 and 409 amino acids in length respectively. It must be noted however that references within the text to numbered residues include the 18 amino acid signal peptide.

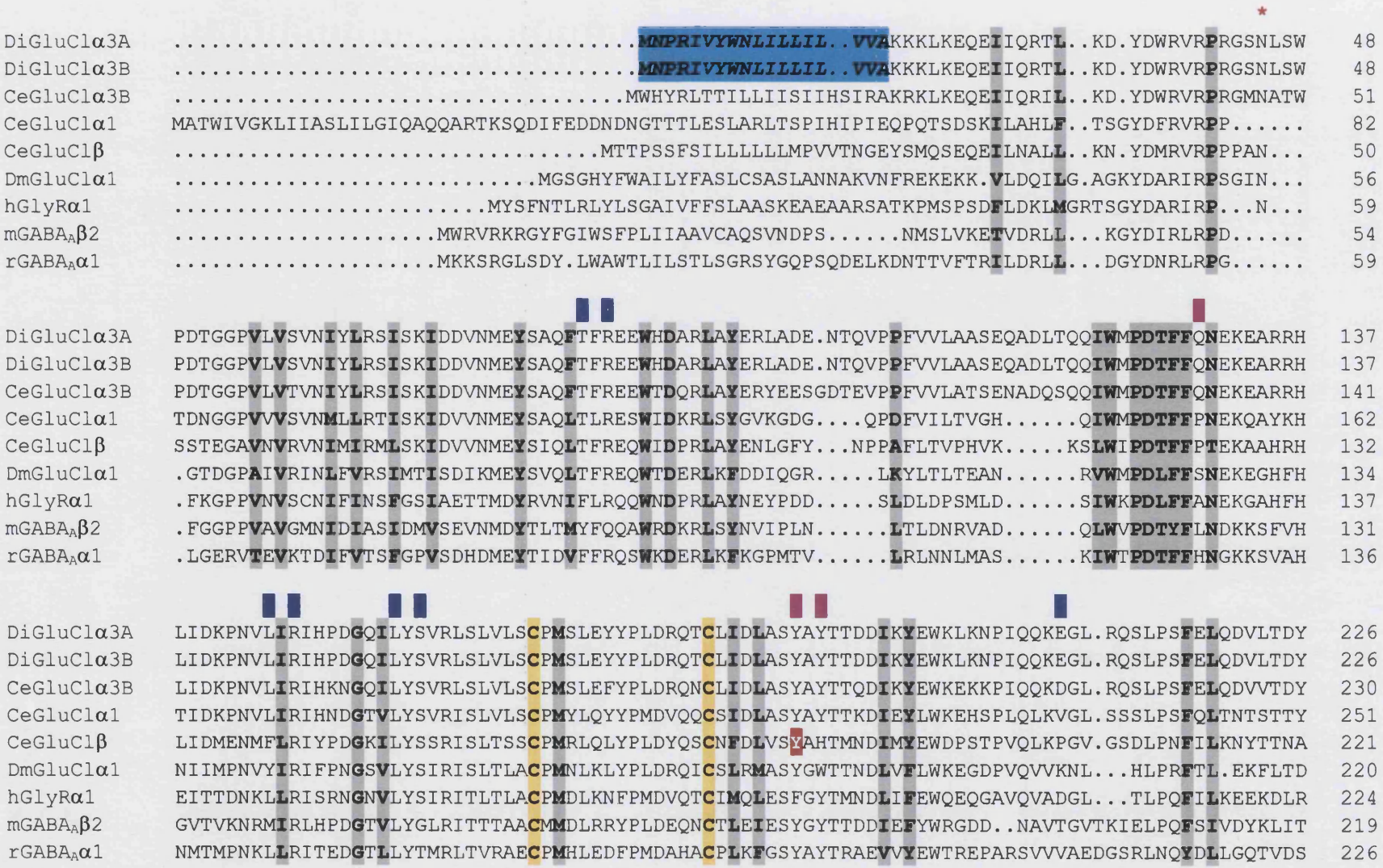
Analysis of the common N-terminal amino acid sequence by the GCG program "Motifs" reveals that Asn-45 is a putative *N*-linked glycosylation site. A highly characteristic feature of the N-terminals of LGICs, the *N*-linked glycosylation site is clearly important for receptor function: mutation of a comparable site (N38A) within the rat glycine α1 subunit abolishes glycine-activated currents (Akagi *et al.*, 1991). However, it is not generally believed that this site plays a role in ligand binding, as could be inferred from this study. Instead, studies on chick $GABA_A$ and *Torpedo* nAChRs, expressed in *Xenopus* oocytes, indicate the putative *N*-linked glycosylation site is crucial for receptor stability and/or efficient insertion of the mature receptors into the plasma membrane (Sumikawa *et al.*, 1988; Gehle & Sumikawa, 1991).

As outlined in the introduction, one of the defining features of the N-terminals of LGIC subunits is a characteristic pair of cysteine residues, which form a disulphide bridge of 15 amino acids. In the *D. immitis* GluClα3 subunits these residues are present at positions 166 and 180. Although accepted not to be part of the agonist-binding site, opinion on the function of the cysteine loop remains polarised. Uncoupling of the loop of nAChRs, by mutating the cysteine residues has been suggested to result in the failure of the subunits to properly assemble (Fu & Sine, 1996; Green & Wanamaker, 1997). However, other groups have suggested the function of the loop is to both provide stability against degradation and

also to determine the efficiency with which the receptors are transported to the cell surface (Blount *et al.*, 1990; Sumikawa & Gehle, 1992). There is a high degree of conservation of amino acids within the loop and mutation of these residues can also affect receptor function. For example, mutation of the glycine-α1 subunit at Asp-148, a residue seemingly conserved across GluCls (Asp-176 in the DiGluClα3 subunits), appears to prevent receptor expression (Vandenberg *et al.*, 1993).

The second cysteine pair, which is indicative of the glycine and GluCl receptors only, emphasising their "orthologous" relationship (Vassilatis *et al.*, 1997b), is located at residues 227 and 238; the length of this disulphide bridge (11 amino acids) is also conserved. The function of this loop has been studied in glycine receptors and it appears that uncoupling the loop or indeed mutating selected residues within it can dramatically affect both subunit assembly and agonist binding (Vandenberg *et al.*, 1992a, 1992b; Rajendra *et al.*, 1995).

The major determinants of agonist binding are contained within the N-terminal of LGIC subunits. Conversely the C-terminals are thought to be largely responsible for forming the ion channel within the membrane, discussed in more detail below. The functional autonomy of these regions has been elegantly demonstrated: the ligand-binding domain of a nACh receptor (N-terminal) has been shown to be capable of activating a 5-HT_3 membrane channel (C-terminal) in a chimeric receptor (Eisele *et al.*, 1993). Similarly, chimeric receptors formed from the GluClα1 (N-terminals) and GluClβ (C-terminals) subunits of *C. elegans* were used to infer that the major determinants for glutamate binding were also present on the N-terminals of GluCls; the homomeric GluClα1 receptors were glutamate insensitive but GluClβ homomers and the chimeric receptors were activated by the agonist. Subsequent site-directed mutagenesis within the pore of GluClα1 revealed this subunit was deficient in coupling ligand binding to channel gating (Etter *et al.*, 1996).


DiGluClα3A
DiGluClα3B
CeGluClα3B
CeGluClα1
CeGluClβ
DmGluClα1
hGlyRα1
mGABA$_A$β2
rGABA$_A$α1

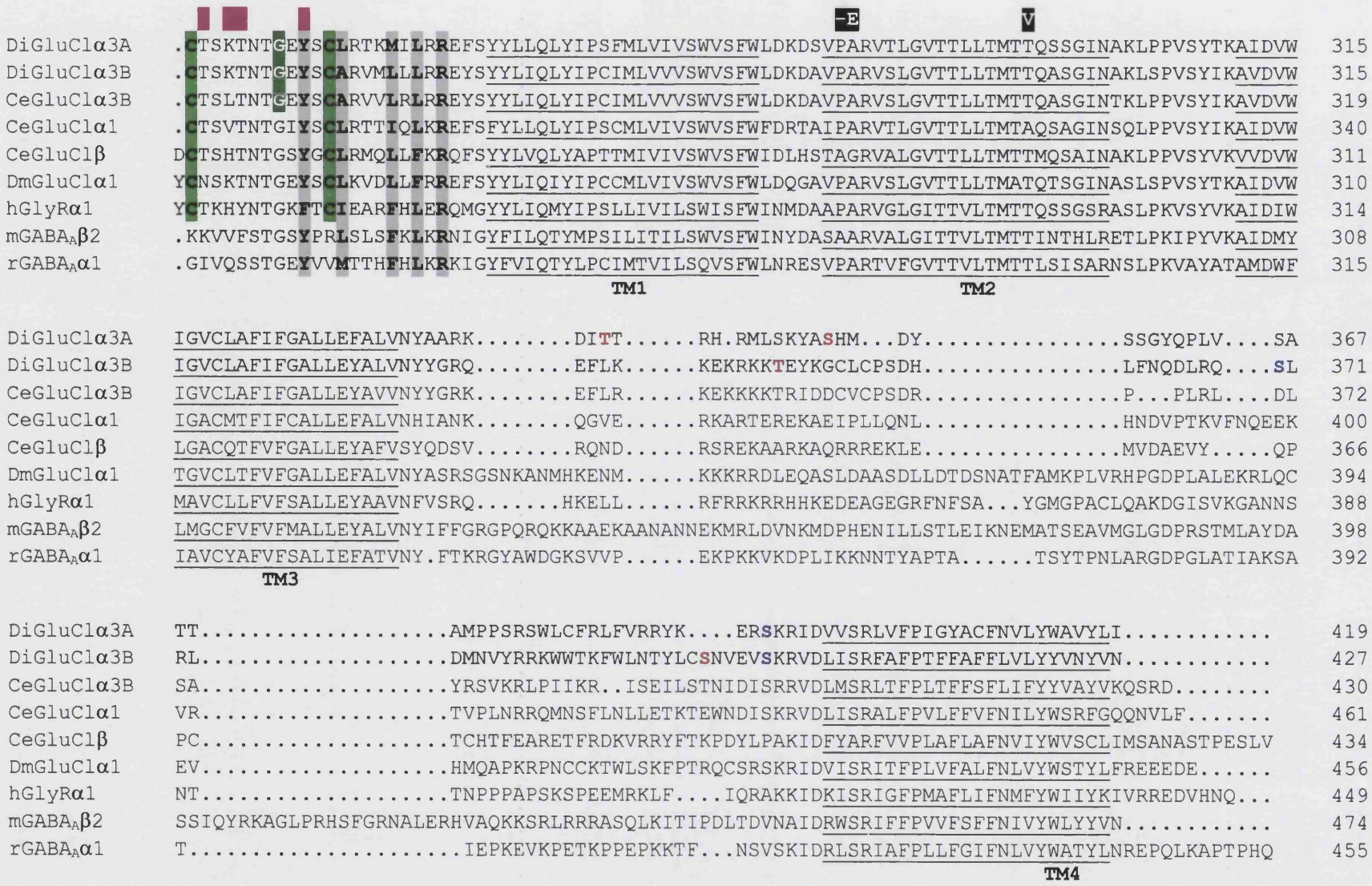

-E
V
DiGluClα3A .CTSKTNTGEYSCLRTKMILRREFSYYLLQLYIPSFMLVIVSWVSFWLDKDSVPARVTLGVTTLLTMTTQSSGINAKLPPVSYTKAIDVW 315
DiGluClα3B .CTSKTNTGEYSCARVMLLLRREYSYYLIQLYIPCIMLVVVSWVSFWLDKDAVPARVSLGVTTLLTMTTQASGINAKLSPVSYIKAVDVW 315
CeGluClα3B .CTSLTNTGEYSCARVVLRLRREYSYYLIQLYIPCIMLVVVSWVSFWLDKDAVPARVSLGVTTLLTMTTQASGINTKLPPVSYIKAVDVW 319
CeGluClα1 .CTSVTNTGIYSCLRTTIQLKREFSFYLLQLYIPSCMLVIVSWVSFWFDRTAIPARVTLGVTTLLTMTAQSAGINSQLPPVSYIKAIDVW 340
CeGluClβ DCTSHTNTGSYGCLRMQLLFKRQFSYYLVQLYAPTTMIVIVSWVSFWIDLHSTAGRVALGVTTLLTMTTMQSAINAKLPPVSYVKVVDVW 311
DmGluClα1 YCNSKTNTGEYSCLKVDLLFRREFSYYLIQIYIPCCMLVIVSWVSFWLDQGAVPARVSLGVTTLLTMATQTSGINASLSPVSYTKAIDVW 310
hGlyRα1 YCTKHYNTGKFTCIEARFHLERQMGYYLIQMYIPSLLIVILSWISFWINMDAAPARVGLGITTVLTMTTQSSGSRASLPKVSYVKAIDIW 314
mGABA$_A$β2 .KKVVFSTGSYPRLSLSFKLKRNIGYFILQTYMPSILITILSWVSFWINYDASAARVALGITTVLTMTTINTHLRETLPKIPYVKAIDMY 308
rGABA$_A$α1 .GIVQSSTGEYVVMTTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFWLNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWF 315
TM1
TM2
DiGluClα3A IGVCLAFIFGALLEFALVNYAARK.....DITT.....RH.RMLSKYASHM...DY.....SSGYQPLV....SA 367
DiGluClα3B IGVCLAFIFGALLEYALVNYYGRQ.....EFLK.....KEKRKKTEYKGCLCPSDH.....LFNQDLRQ....SL 371
CeGluClα3B IGVCLAFIFGALLEYAVVNYYGRK.....EFLR.....KEKKKKTRIDDCVCPSDR.....P...PLRL....DL 372
CeGluClα1 IGACMTFIFCALLEFALVNHIANK.....QGVE.....RKARTEREKAEIPLLQNL.....HNDVPTKVFNQEEK 400
CeGluClβ LGACQTFVFGALLEYAFVSYQDSV.....RQND.....RSREKAARKAQRRREKLE.....MVDAEVY.....QP 366
DmGluClα1 TGVCLTFVFGALLEFALVNYASRSGSNKANMHKENM.....KKKRRDLEQASLDAASDLLDTDSNATFAMKPLVRHPGDPLALEKRLQC 394
hGlyRα1 MAVCLLFVFSALLEYAAVNFVSRQ.....HKELL.....RFRRKRRHHKEDEAGEGRFNFSA...YGMGPACLQAKDGISVKGANNS 388
mGABA$_A$β2 LMGCFVFVFMALLEYALVNYIFFGRGPQRQKKAAEKAANANNEKMRLDVNKMDPHENILLSTLEIKNEMATSEAVMGLGDPRSTMLAYDA 398
rGABA$_A$α1 IAVCYAFVFSALIEFATVNY.FTKRGYAWDGKSVVP.....EKPKKVKDPLIKKNNTYAPTA.....TSYTPNLARGDPGLATIAKSA 392
TM3
DiGluClα3A TT.....AMPPSRSWLCFRLFVRRYK....ERSKRIDVVSRLVFPIGYACFNVLYWAVYLI..... 419
DiGluClα3B RL.....DMNVYRRKWWTKFWLNTYLCSNVEVSKRVDLISRFAFPTFFAFFLVLYYVNYVN..... 427
CeGluClα3B SA.....YRSVKRLPIIKR..ISEILSTNIDISRRVDLMSRLTFPLTFFSFLIFYYVAYVKQSRD..... 430
CeGluClα1 VR.....TVPLNRRQMNSFLNLLETKTEWNDISKRVDLISRALFPVLFFVFNILYWSRFGQQNVLF..... 461
CeGluClβ PC.....TCHTFEARETFRDKVRRYFTKPDYLPAKIDFYARFVVPLAFLAFNVIYWVSCLIMSANASTPESLV 434
DmGluClα1 EV.....HMQAPKRPNCCKTWLSKFPTRQCSRSKRIDVISRITFPLVFALFNLVYWSTYLFREEEDE..... 456
hGlyRα1 NT.....TNPPPAPSKSPEEMRKLF....IQRAKKIDKISRIGFPMAFLIFNMFYWIIYKIVRREDVHNQ... 449
mGABA$_A$β2 SSIQYRKAGLPRHSFGRNALERHVAQKKSRLRRRASQLKITIPDLTDVNAIDRWSRIFFPVVFSFFNIVYWLYYVN..... 474
rGABA$_A$α1 T.....IEPKEVKPETKPPEPKKTF...NSVSKIDRLSRIAFPLLFGIFNLVYWATYLNREPQLKAPTPHQ 455
TM4

Figure 3.10. Alignment of the proposed DiGluClα3 amino acid sequences with other LGCC subunits. The *D. immitis* (Di) GluClα3 subunits amino acid sequences were aligned with selected GluCl subunits from *C. elegans* (Ce) and *D. melanogastor* (Dm), and representative mammalian (h=human; m=mouse; r=rat) LGCC subunits using MultAlin (Corpet, 1988). Characteristic features of all the subunits are the signature pair of cysteine residues (highlighted in yellow) and TMs1-4 (underlined). The cysteine residues, indicative of the GluCls and glycine receptors, are highlighted in light green. Features of the DiGluClα3 subunits are the 18 amino acid signal peptides (light blue highlighted italics), the *N*-linked glycosylation site (red star) and the potential regulatory phosphorylation sites between TM3-4 for protein kinases (red font=casein kinase II; blue font=protein kinase C; pink font=cAMP dependent protein kinase). The proposed alternative splicing site for the GluClα3 subunits is highlighted in dark green, the glycine residue marking the beginning of the differing C-terminals. The residues highlighted in grey have been proposed to be highly conserved across the superfamily and are thought to form the core structure of the LBD (Brejc *et al.*, 2001). The blue and pink boxes above the text indicate the positions of the residues implicated to form the "loops" that form the binding site in nACh receptors (pink="loops" A-C; blue="loops" D-F) (Brejc *et al.*, 2001; Sine, 2002). Tyrosine-182 of the CeGluClβ subunit, which is implicated in glutamate binding, is highlighted in red (Li *et al.*, 2002; Slimko & Lester, 2003). Highlighted in black above the TM2 region are the conserved residues found in nACh and 5-HT_{3A} receptor subunits at the equivalent positions that confer their cation selectivity; the dash indicates the absence of the proline residue in these sequences (Galzi *et al.*, 1992; Keramidas *et al.*, 2000; Gunthorpe & Lummis, 2001).

The ligand-binding domain (LBD) has been extensively studied in nAChRs using a variety of approaches (reviewed in Karlin, 2002 & Sine, 2002). Binding has been determined to occur at a "pocket" formed at the interface between selected subunits. The α- subunits are thought to form the principle part of the binding site contributing the so-called "loops" A-C whilst the neighbouring subunits contribute the "loops" D-F, the combined 3-dimensional arrangement forming the "pocket". There is not absolute conservation of residues proposed to form the binding "loops" between subunits, possibly explaining, in part, why certain receptors and indeed, "pockets" within the receptors have differing affinities for agonist. As the nAChα7 subunit forms homomeric receptors each polypeptide contains all the residues responsible for forming "loops" A-F. The positions of the key agonist binding residues are indicated in figure 3.10. Crystallisation of a molluscan acetylcholine binding protein also identified a number of residues that appear to form the core structure of the LBD, bringing these loops together (Brejc *et al.*, 2001). These, mainly, hydrophobic amino acids exhibit a high degree of conservation across the superfamily (also highlighted in figure 3.10). It has therefore been suggested that all LGICs have a similar LBD configuration (Brejc *et al.*,

2001), although little structural evidence exists to confirm such an assertion for other classes of receptor.

Site-direct mutagenesis studies on $GABA_A$ subunits have revealed a number of amino acids, which appear to be important for agonist binding to these receptors. As for all mutagenesis studies, it is sometimes difficult to be certain that the mutated residues are directly involved in binding or have a distant, indirect effect, which may still alter the activation of the receptor. However, significant effects on agonist binding and channel activation have been observed within $GABA_A$ receptors following mutations of residues that either align with the proposed binding or key structural amino acids of nAChR subunits (reviewed in Chang & Weiss, 2000). For example, mutations within the rat $GABA_A$ α1 subunit at positions F64 and R66 that correspond to residues T81 and R83 of the *D. immitis* GluClα3 subunits and those residues, which form "loop" D of the binding site in nAChRs (contributed by the β and α7 subunits), have lead to 73-320 fold increases in the GABA EC_{50} (Sigel *et al.*, 1992; Smith & Olsen, 1994; Boileau *et al.*, 1999). Similarly, mutation of Y157 of the rat $GABA_A$β2 subunit or the equivalent (in position) Y198 of the human $GABA_A$ρ1 subunit also affects the GABA EC_{50} significantly (Amin & Weiss, 1993). These latter positions correspond to a tryptophan residue within "loop" B of the nAChRs (contributed by the α subunits) and indeed a conserved tyrosine (position 186 of the DiGluClα3 subunits) within GluCl subunits. Interestingly, this tyrosine is the only amino acid residue so far determined to play a key role in glutamate binding to GluCls. Mutation of this residue in *C. elegans* GluClβ subunits (tyr-182) to a phenylalanine reduced the glutamate response of the GluClα1β heteromers to 16%. During these studies a number of other residues predicted to lie within "loop" regions of GluClβ were mutated. Although many lead to dramatic effects on receptor activation by glutamate it was difficult to discern whether these were truly due to effects on the binding site or not (Li *et al.*, 2002; Slimko & Lester, 2003). Should the structure of the binding sites be conserved across the superfamily, as highlighted by the above examples, it is tempting to hypothesise that the $GABA_A$α subunits may contribute the equivalent "loops" to those D-F found in the nAChβ subunits where as the $GABA_A$β and ρ subunits may contribute the equivalent "loops" A-C found in nAChα subunits. In addition, as most GluCl subunits examined so far are capable of

forming homomeric receptors this suggests all the determinants or "loops" for ligand binding must be typically contained within each polypeptide, as described for nAChα7 receptors.

The C-terminal of the *D. immitis* GluClα3 subunits, simply from alignment with other GluCl subunits, clearly exhibits the four characteristic, hydrophobic TM regions; hydropathy plots for each subunit confirm their presence (see appendix D). Residues that comprise TM1-3 are highly conserved across the LGIC superfamily, particularly within each class of receptor, where as TM4 varies more considerably in sequence. The TMs were classically viewed as four α-helices although increasing evidence suggests that only TM2 and probably TM4 exhibit such secondary structure. A recent study focusing on glycine receptor subunits proposed a model where by the classical TM1 is actually three membrane spanning beta strands where as TM3 has a mixed single membrane spanning structure, which may only have partial helical character (Leite & Cascio, 2001). Extensive work on the nACh receptor structure also argues against the classical four α-helical nature of the TMs (Unwin, 1993). The second TM of each subunit lines the membrane channel, residues within this region being implicated in channel gating and ion selectivity. Where as the actual residues responsible for forming the gate remain somewhat controversial, although they appear to be located between the middle to cytoplasmic face of TM2 (Xu *et al.*, 1995; Xu & Akabas, 1996; Chang *et al.*, 1996; Chang & Weiss, 1998), it has been well established that only three amino acid mutations within this region are required to change the ion selectivity of the channel (highlighted in figure 3,10). Both α7 nACh and 5-HT_{3A} receptors can be made anion selective by changing a valine to a threonine, a glutamate to an alanine (neutralising a ring of negative charge) and inserting an "extra" amino acid (proline), which is not present in these channels (the positions of these mutations are highlighted in figure 3.10) (Galzi *et al.*, 1992; Gunthorpe & Lummis, 2001). The reverse changes in glycine receptors bestow cation selectivity (Keramidas *et al.*, 2000); given the sequence conservation this may also be possible for GluCls.

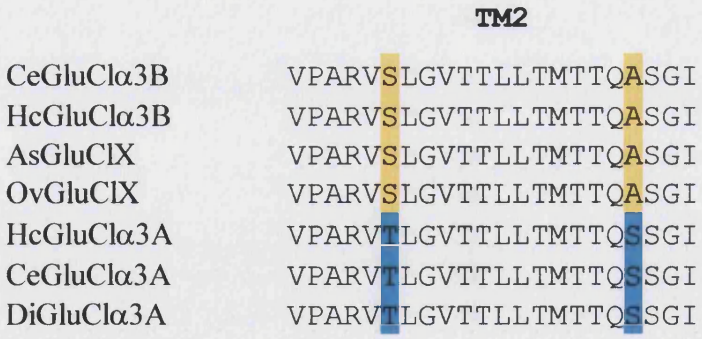


Figure 3.11. Alignment of the TM2 domains of the GluClα3 subunits. The TM2 regions of nematode GluClα3 subunits were aligned using the GCG program "pileup". The two-letter prefix on each subunit denotes the species of nematode: Ce (*C. elegans*); Hc (*H. contortus*); As (*Ascaris suum*); Ov (*Onchocerca volvulus*); Di (*D. immitis*). The GluClX sequences are so called because they are derived from partial GluClα3B cDNAs. The highlighted in yellow are the conserved residues consistently found in GluClα3B-type subunits where as in blue are those found in the GluClα3A type subunits.

There are notable conserved differences in amino acid sequence at selected positions between the GluClα3A and α3B subunit across species. As the N-terminals are identical, these key differences all occur in the C-terminals of these polypeptides. Although little is known about the functional roles of individual amino acids in GluCls, it is tempting to speculate that these conserved differences may well be important in explaining different properties between the two alternatively spliced subunit classes. For example, CeGluClα3B can form glutamate-sensitive, homomeric channels in *Xenopus* oocytes where as the CeGluClα3A subunits, despite seemingly having the same LBD cannot (Dent *et al.*, 2000). Figure 3.11 highlights such differences with the TM2 domains of the alternatively spliced forms. Towards the intracellular side of the TM, GluClα3A and GluClα3B subunits exhibit conserved threonine and serine residues respectively. Interestingly, it is at this position in the GluClα1 subunit of *C. elegans* that the threonine residue can be located, which when mutated to an alanine, glycine, or proline, changed the ability of the homomeric GluClα1 receptor to be gated by glutamate. However it must be noted that the conservative change to a serine residue (the difference observed between the GluClα3 spliced forms) did not appear to alter the channel properties (Etter *et al.*, 1996). Mutations in the equivalent position in the *Drosophila melanogastor* RDL subunit (a GABA receptor subunit) can

cause dieldrin- and picrotoxin-resistance (Ffrench-Constant *et al.*, 1993). No evidence exists to speculate on the effect of the second conserved change in the TM2 domains.

The large intracellular loop between TM3 and TM4 is the most variable region between the two subunits with little amino acid conservation. This is a common feature amongst closely related members of LGIC sub-families and presumably relates to the differential regulation of potentially otherwise similar polypeptides. In support of this are a number of putative phosphorylation sites within the loop regions of these subunits, as predicted by the GCG program "Motfis". The DiGluClα3A subunit has two predicted casein kinase II (CKII) phosphorylation sites at T342 and S354 and one potential protein kinase C (PKC) site at S392. In contrast the intracellular loop of the GluClα3B subunit has two predicted PKC sites at S370 and S399, a CKII site at S394 and a cAMP-dependent phosphorylation site at T350. Whether all these sites are genuine substrates for protein kinases is unknown, as no studies have been conducted into the regulation of GluCls. In other classes of LGIC subunits numerous studies have indicated how important phosphorylation to regulating receptor function is, affecting the amplitude of agonist activated currents, the rate of desensitisation and the clustering of receptors (Porter *et al.*, 1990; Wafford & Whiting, 1992; Leidenheimer *et al.*, 1993; Krishek *et al.*, 1994; McDonald & Moss, 1994, 1997; Macdonald, 1995; Moss *et al.*, 1995, 1996; McDonald *et al.*, 1998; Filippova *et al.*, 2000).

3.3 Results and Discussion 2:
Isolation of a novel LGIC subunit from *D. immitis*

3.3.1 Amplification of a putative LGIC subunit cDNA by degenerate PCR

As outlined in the introduction, one of the initial aims of the project was to amplify additional (to *avr-14*) *D. immitis* GluCl subunit cDNAs by PCR, using degenerate oligonucleotide primers. An alignment of all available GluCl amino acid sequences was constructed and conserved regions were identified for the α-type and β-type subunits (see appendix C). Oligonucleotide primers, with degeneracy limited throughout the sequence to less than 1000-fold, were subsequently designed to these regions. In an attempt to enhance specificity during annealing and limit the overall degeneracy of the primer, the 3' ends of the oligonucleotides were designed, if possible, to amino acids encoded by limited codons, e.g. methionine. Asymmetric codon use, i.e. the preference for one codon over another to encode the same amino acid, is evident between different species of nematode and was identified as another way of limiting the degeneracy of the primers. Filarial nematodes exhibit a significant AT (adenine-thymine) bias in their genomes (Rothstein *et al.*, 1988; Fadiel *et al.*, 2001), being most clearly evident in coding regions at the third position of codons. A codon frequency chart (appendix E) was therefore constructed from 15 *D. immitis* mRNA sequences, using the GCG program "CodonFrequency". Using this chart, the codons for particular residues, if possible or indeed if necessary to limit the degeneracy, could be predicted.

Nucleotide details of the nine degenerate primers designed can be found in appendix A. Of these, six were designed to conserved motifs shared between the GluClα1 and α2 subunits of *C. elegans* and three were designed to motifs shared between the GluClβ subunits of *C. elegans* and *H. contortus* (highlighted in appendix C). A large number of primer combinations were attempted using both conventional 3-step and Touchdown approaches to PCR. Limited success, leading to unexpected results, were had using two anti-sense primers, designed to the highly conserved WxPDTFF motif, in conjunction with the SL1 primer; these primers ASDGAP2 and ASDGBP2 were biased in design to the α- and β-type subunits respectively. The ASDGAP2/SL1 PCR produced three bands (150, 250, and

450bp) where as the ASDGBP2/SL1 PCR produced four bands (250, 2x450, and 600bp) as shown in figure 3.12.

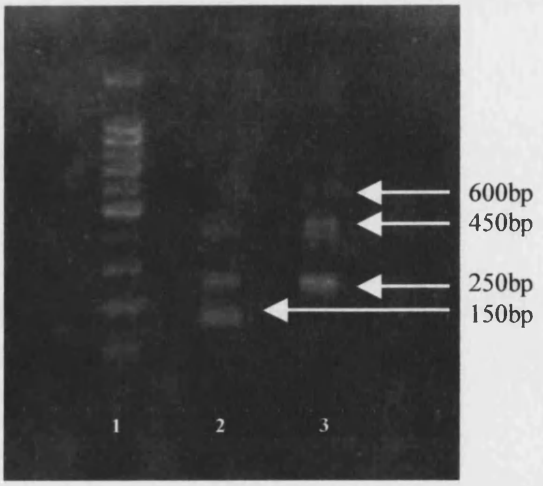


Figure 3.12. Amplified products of degenerate PCR using ASDGAP2 and ASDGBP2 in tandem with SL1. Lane 1: 1kb DNA ladder; lane 2: Products amplified following PCR using the primers ASDGAP2 and SL1; Lane 3: Products amplified following PCR using the primers ASDGBP2 and SL1. Both PCRs used 3-step programs with annealing temperatures of 50°C.

All the bands were cloned using pCR®-Blunt II-TOPO as before, recombinant plasmids being identified by restriction digest with EcoRI. These recombinants were then sequenced using either M13 primer. Clones of the larger bands generated from the ASDGAP2/SL1 PCR and the 250bp product of the ASDGBP2/SL1 PCR were identified as partial *avr-14* sequences, the differences in size appearing to be due to the mis-annealing of the SL1 primer. The product of interest however, was the larger of the two bands at ~450bp from the ASDGBP2/SL1 PCR.

Analysis of the 450bp cDNA sequence revealed a putative start codon, 44 nucleotides downstream of the SL1 sequence, and a partial open reading frame that when translated exhibited 52% amino acid identity to a predicted LGIC subunit in *C. elegans* (T27A1.4). The clone also exhibited weaker identity (20-30%) with various GluCl, glycine and $GABA_A$ subunits. The full-length coding region was deduced following 3' RACE, which was achieved, using a sense primer (SBETA2P2) designed to the partial cDNA sequence in tandem with the RoRi primer. The PCR product of 1.2kb (figure 3.13A) was cloned as before and sequenced. From the mRNA sequence, predicted from the overlapping cDNAs, full-length primers (SDG1 and ASDG1) were designed to amplify the proposed 1221 nucleotide coding region. Figure 3.13B shows a band of the expected size was amplified, which was subsequently cloned and fully sequenced on both strands. The sequence of this cDNA was identical to that compiled from the overlapping clones and was therefore

adopted as the consensus sequence for the coding region. When translated, this sequence exhibited amino acid identity to other LGICs comparable to that observed for the partial cDNA. It was therefore proposed that the 1221 nucleotide open reading frame (1362 nucleotide mRNA) encoded a 406 amino acid, novel LGIC from *D. immitis*, termed DG1.

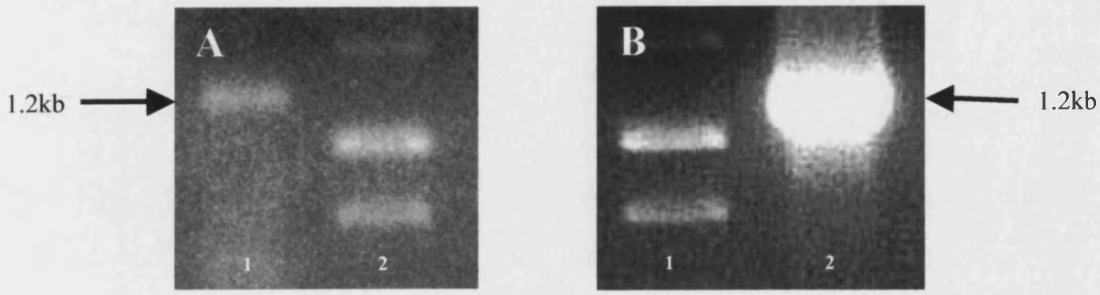


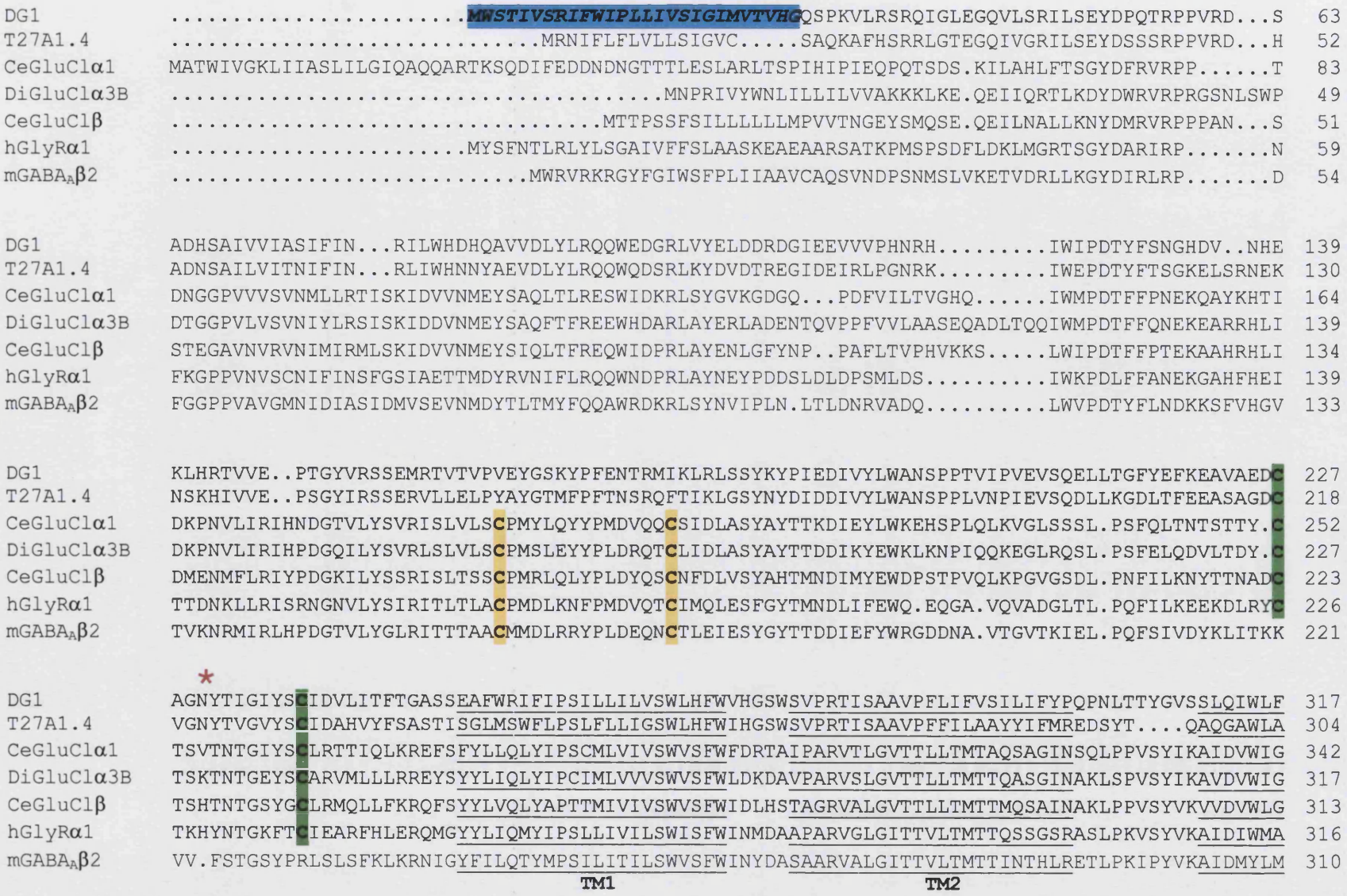
Figure 3.13. Amplification of DG1 cDNAs. Lane 1; 1kb DNA ladder; lane 2 PCR product. Each PCR used a 3-step PCR program; **A: 3' RACE PCR** (primers SBETAP2 & RoRi; annealing temperature of 51°C); **B: Full-length coding region PCR** (primers SDG1 & ASDG1; annealing temperature of 51°C).

3.3.2 Sequence analysis of DG1

As stated earlier, the amino acid sequence of DG1 exhibits 52% identity with T27A1.4 (a predicted LGIC in *C. elegans*) and 20-30% identity with other LGCC subunits. This level of homology is sufficient to indicate that DG1 could be a member of the LGIC superfamily; GluCls and vertebrate $GABA_A$ receptor subunits share ~30% identity. Further analysis of the subunit's amino acid sequence reveals it is highly unusual (figure 3.14) and although certain characteristic motifs of the LGIC superfamily are present, others are surprisingly absent. The program SignalP V1.1 (Nielsen *et al.*, 1997) indicates the putative subunit to have a signal peptide of 27 amino acids making the mature polypeptide, following cleavage, to be 379 residues in length, short compared to other LGIC subunits. Within the N-terminal a number of residues implicated to form the core LBD structure appear to be conserved in both DG1 and T27A1.4, e.g. the WxPD(T/L)(F/Y)F motif. Strikingly however, the pair of cysteine residues that form the signature disulphide bridge is absent. Curiously, the second pair of cysteines that form the characteristic loop observed in GluCls and glycine receptors only, is present at the conserved distance of 11 amino acid residues.

In addition, within the loop lies a potential *N*-linked glycosylation site, as predicted by the GCG program "Motifs"

Analysis of the C-terminal reveals little sequence identity across the proposed TM regions between DG1 and the LGCCs, although there is considerable identity with T27A1.4. A hydropathy plot for DG1 however, indicates four hydrophobic regions that align almost perfectly with the predicted TMs of other subunit classes. The proposed TM2 domains of DG1 and T27A1.4 are highly unusual, not least because of the presence of a proline residue in the middle of this region. Indeed, the inclusion of such an amino acid may be predicted to significantly distort the proposed α-helical character of TM2. Between the TM3 and TM4 lies the intracellular loop, the length of which for DG1 and indeed T27A1.4 accounting for their overall comparably short nature. Within the loop, as for other LGICs, there are potential sites for regulation by phosphorylation (predicted by the GCG program "Motifs"): PKC and Tyrosine kinase sites exist at positions 341 and 361 respectively.


DG1 .....MWSTIVSRIFWIPLLIVSIGIMVTVHGQSPKVLRSRQIGLEGQVLSRILSEYDPQTRPPVRD...S 63
T27A1.4 .....MRNIFLFLVLLSIGVC.....SAQKAFHSRRLGTEGQIVGRILSEYDSSSRPPVRD...H 52
CeGluClα1 MATWIVGKLIIASLILGIQAQQARTKSQDIFEDDNDNGTTTLESLARLTSPIHIPIEQPQTSDS.KILAHLFTSGYDFRVRPP.....T 83
DiGluClα3B .....MNPRIVYWNLILLILVVAKKKLKE.QEIIQRTLKDYDWRVRPRGSNLSWP 49
CeGluClβ .....MTTPSSFSILLLLLLMPVVTNGEYSMQSE.QEILNALLKNYDMRVRPPPAN...S 51
hGlyRα1 .....MYSFNTLRLYLSGAIVFFSLAASKEAEAARSATKPMSPSDFLDKLMGRTSGYDARIRP.....N 59
mGABA$_A$β2 .....MWRVRKRGYFGIWSFPLIIAAVCAQSVNDPSNMSLVKETVDRLLKGYDIRLRP.....D 54
DG1 ADHSAIVVIASIFIN...RILWHDHQAVVDLYLRQQWEDGRLVYELDDRDGIEEVVVPHNRH.....IWIPDTYFSNGHDV..NHE 139
T27A1.4 ADNSAILVITNIFIN...RLIWHNNYAEVDLYLRQQWQDSRLKYDVDTREGIDEIRLPGNRK.....IWEPDTYFTSGKELSRNEK 130
CeGluClα1 DNGGPVVVSVNMLLRTISKIDVVNMEYSAQLTLRESWIDKRLSYGVKGDGQ...PDFVILTVGHQ.....IWMPDTFFPNEKQAYKHTI 164
DiGluClα3B DTGGPVLVSVNIYLRSISKIDDVNMEYSAQFTFREEWHDARLAYERLADENTQVPPFVVLAASEQADLTQQIWMPDTFFQNEKEARRHLI 139
CeGluClβ STEGAVNVRVNIMIRMLSKIDVVNMEYSIQLTFREQWIDPRLAYENLGFYNP..PAFLTVPHVKKS.....LWIPDTFFPTEKAAHRHLI 134
hGlyRα1 FKGPPVNVSCNIFINSFGSIAETTMDYRVNIFLRQQWNDPRLAYNEYPDDSLDLDPSMLDS.....IWKPDLFFANEKGAHFHEI 139
mGABA$_A$β2 FGGPPVAVGMNIDIASIDMVSEVNMDYTLTMYFQQAWRDKRLSYNVIPLN.LTLDNRVADQ.....LWVPDTYFLNDKKSFVHGV 133
DG1 KLHRTVVE..PTGYVRSSEMRTVTVPVEYGSKYPFENTRMIKLRLSSYKYPIEDIVYLWANSPPTVIPVEVSQELLTGFYEFKEAVAEDC 227
T27A1.4 NSKHIVVE..PSGYIRSSERVLLELPYAYGTMFPFTNSRQFTIKLGSYNYDIDDIVYLWANSPPLVNPIEVSQDLLKGDLTFEEASAGDC 218
CeGluClα1 DKPNVLIRIHNDGTVLYSVRISLVLSCPMYLQYYPMDVQQCSIDLASYAYTTKDIEYLWKEHSPLQLKVGLSSSL.PSFQLTNTSTTY.C 252
DiGluClα3B DKPNVLIRIHPDGQILYSVRLSLVLSCPMSLEYYPLDRQTCLIDLASYAYTTDDIKYEWKLKNPIQQKEGLRQSL.PSFELQDVLTDY.C 227
CeGluClβ DMENMFLRIYPDGKILYSSRISLTSSCPMRLQLYPLDYQSCNFDLVSYAHTMNDIMYEWDPSTPVQLKPGVGSDL.PNFILKNYTTNADC 223
hGlyRα1 TTDNKLLRISRNGNVLYSIRITLTLACPMDLKNFPMDVQTCIMQLESFGYTMNDLIFEWQ.EQGA.VQVADGLTL.PQFILKEEKDLRYC 226
mGABA$_A$β2 TVKNRMIRLHPDGTVLYGLRITTTAACMMDLRRYPLDEQNCTLEIESYGYTTDDIEFYWRGDDNA.VTGVTKIEL.PQFSIVDYKLITKK 221
DG1 AGNYTIGIYSCIDVLITFTGASSEAFWRIFIPSILLILVSWLHFWVHGSWSVPRTISAAVPFLIFVSILIFYPQPNLTTYGVSSLQIWLF 317
T27A1.4 VGNYTVGVYSCIDAHVYFSASTISGLMSWFLPSLFLLIGSWLHFWIHGSWSVPRTISAAVPFFILAAYYIFMREDSYT....QAQGAWLA 304
CeGluClα1 TSVTNTGIYSCLRTTIQLKREFSFYLLQLYIPSCMLVIVSWVSFWFDRTAIPARVTLGVTTLLTMTAQSAGINSQLPPVSYIKAIDVWIG 342
DiGluClα3B TSKTNTGEYSCARVMLLLRREYSYYLIQLYIPCIMLVVVSWVSFWLDKDAVPARVSLGVTTLLTMTTQASGINAKLSPVSYIKAVDVWIG 317
CeGluClβ TSHTNTGSYGCLRMQLLFKRQFSYYLVQLYAPTTMIVIVSWVSFWIDLHSTAGRVALGVTTLLTMTTMQSAINAKLPPVSYVKVVDVWLG 313
hGlyRα1 TKHYNTGKFTCIEARFHLERQMGYYLIQMYIPSLLIVILSWISFWINMDAAPARVGLGITTVLTMTTQSSGSRASLPKVSYVKAIDIWMA 316
mGABA$_A$β2 VV.FSTGSYPRLSLSFKLKRNIGYFILQTYMPSILITILSWVSFWINYDASAARVALGITTVLTMTTINTHLRETLPKIPYVKAIDMYLM 310
TM1
TM2

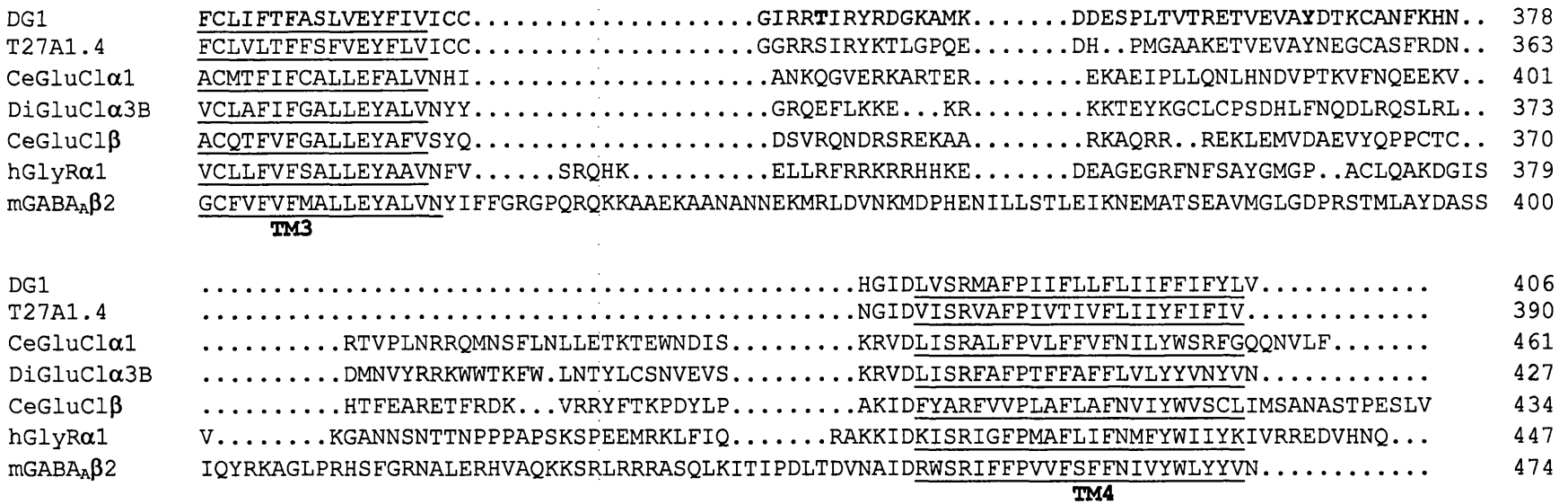


Figure 3.14. Alignment of the proposed DG1 amino acid sequence with T27A1.4 and selected LGCC subunits. The DG1 subunit amino acid sequence was aligned with the predicted LGIC subunit T27A1.4 from *C. elegans,* selected nematode GluCl subunits (Ce=*C. elegans*; Di=*D. immitis*), and representative mammalian (h=human; m=mouse; r=rat) LGCC subunits using MultAlin (Corpet, 1988). The pair of cysteine residues indicative of the LGIC superfamily (highlighted in yellow) appear to be absent in DG1 and T27A1.4, although the cysteine pair indicative of the GluCls and glycine receptors (highlighted in green) is conserved in these putative subunits. Features of the DG1 subunit are the 27 amino acid signal peptide (light blue highlighted italics), the *N*-linked glycosylation site (red star) and two potential regulatory phosphorylation sites between TM3-4 for protein kinases (blue font=protein kinase C; orange font=tyrosine kinase).

3.3.3 RNA interference using T27A1.4

As an aside to the major goal of the project, i.e. the investigation of *D. immitis* GluCl pharmacology, a number of preliminary RNA interference (RNAi) experiments were conducted in an attempt to ascertain a function for the predicted LGIC subunit T27A1.4. It was hoped that progress in ascertaining a potential function for this protein would direct subsequent work on DG1.

RNAi is the selective down regulation ("knocking-down") of a target mRNA by the introduction of homologous double-stranded RNA (dsRNA). Pioneered in *C. elegans*, the RNAi phenomenon has also been used to study gene function in, amongst others, fruit flies, plants and increasingly mammalian cells. Essentially the introduced dsRNA is cleaved into small interfering RNAs (siRNAs; 21-23 nucleotides in length), which then associate with a nuclease complex to direct the selective degradation of the target mRNA (Hannon, 2002). Delivery of the dsRNA into *C. elegans* can be achieved using several methods including microinjection and indeed, soaking the worms in a dsRNA solution. However, the method used here was that of feeding (Fire *et al.*, 1998), where the worms ingest dsRNase-deficient bacteria that are expressing the dsRNA corresponding to the gene of interest, i.e. T27A1.4. The dsRNA is generated by transcription from opposing T7 primers located externally to the multiple cloning-site, on opposite strands, of the L4440 "feeding" vector. Following a period of incubation, the worms were then analysed for gross phenotypes.

Before conducting the experiments a T27A1.4 cDNA had to be cloned. Template (for PCR) cDNA was synthesised from *C. elegans* mRNA, which had previously been isolated from extracted total RNA, as described in section 2.2.5.2. Meanwhile, oligonucleotide primers (ST27A1.4 and AST27A1.4) were designed to amplify the predicted coding region of T27A1.4. A 3-step PCR (annealing temperature of 50°C) using these primers amplified a band of the expected size (~1.2kb), which was cloned into pCR®-Blunt II-TOPO. A recombinant clone, identified by restriction digest with EcoRI, was subsequently sequenced with the M13 primers, the resulting sequence confirming that deposited in the database. The cDNA was then sub-cloned (at the PstI/SacI sites) into the L4440 plasmid (figure 3.15). Finally, this construct was transformed into the (dsRNase deficient) HT115 *E. coli*

strain, used in the experiments. The RNAi experiments were then conducted as described in section 2.2.6.2.

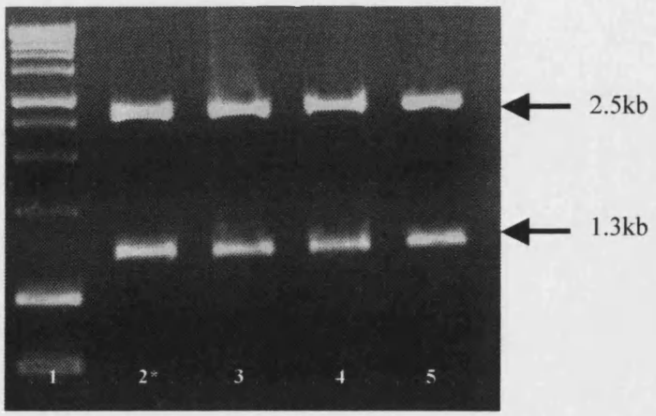


Figure 3.15. Restriction analysis of potential recombinant L4440/T27A1.4 clones. Lane 1: 1kb DNA ladder; lanes 2-5: individual clones screened by restriction digestion with PstI/SacI. All four clones were positive, containing the L4440 band at 2.5kb and the T27A1.4 insert at 1.2kb. The asterisk indicates the recombinant clone used in the RNAi experiments.

The RNAi experiments were repeated three-times but on each occasion no obvious gross phenotype was observed. The experimental procedure however, was validated by the strong characteristic twitching phenotype displayed in the *unc-22* control. Although a positive result (i.e. a gross phenotype) from these experiments may have been very revealing it is difficult to draw many conclusions from such an apparently unaffected outcome. It is entirely possible that "knocking-down" this gene has no real effect on the nematode under lab conditions. Alternatively, it is entirely plausible that such a crude assay was missing subtle phenotypes displayed by the worms, e.g. modulation of pharyngeal pumping or foraging behaviour.

3.4 Summary

In summary, the cloning aspect of this study has proved largely successful: two *D. immitis* GluCls cDNAs and one, potentially novel LGIC cDNA, DG1, have been amplified. The high degree of amino acid identity across orthologous GluClα3 subunits suggests similar pharmacological properties, the study of which formed the basis for the remainder of the investigation. DG1 and indeed, T27A1.4 may represent outlying members of the LGIC superfamily; the contrasting conserved nature of certain characteristic features of these channels (i.e. second "cys-loop" and apparent 4 TM regions) against the absence of such a defining feature as the first "cys-loop" remains a conundrum. Further investigation into the functional roles of these putative subunits, although not pursued here, is certainly warranted. If it could be established that they were important for the worm's viability, their highly unusual nature may make them promising drug targets.

Chapter 4

Expression of the DiGluClα3 Subunits in Mammalian Cell-Lines

4.1 Introduction

Once the GluClα3 subunit cDNAs had been cloned, the objective of the study became to investigate their pharmacology, in particular establishing whether either polypeptide was a molecular target for the AMs. If such a target could be identified, the question of why these drugs are so effective against the larval stages of *D. immitis*, compared to the adult worms, could begin to be addressed. In addition, from a commercial perspective, should either subunit be viably expressed *in vitro* then screens may be developed to identify new or modified existing compounds that exhibit high affinity interactions or activity towards them.

The initial approach proposed to investigate the pharmacology was to perform [^{3}H]-IVM binding assays using membranes prepared from a mammalian cell-line transiently expressing the individual DiGluClα3 subunits. Using a similar method, the *H. contortus* GluClα and GluClα3B subunits had previously been shown to bind [^{3}H]-IVM with K_D values of 26-110pM and 70pM respectively (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002), figures that could account for [^{3}H]-IVM binding to whole worm membrane preparations (K_D from 0.07-0.6nM) (Rohrer *et al.*, 1994; Hejmadi *et al.*, 2000; Cheeseman *et al.*, 2001). These values were also comparative to the high affinity IVM binding site described in *C. elegans* (K_D = 0.26nM) (Schaeffer & Haines, 1989) and the concentrations of drug that exhibit anthelmintic activity in both species. The implications from these studies are that the GluCls are the molecular targets through which these compounds act. The recombinant HcGluClα3A and HcGluClβ subunits exhibited no specific [^{3}H]-IVM binding (Cheeseman *et al.*, 2001). These binding data were largely in agreement with investigations using *Xenopus* oocytes expressing recombinant GluCls from *C. elegans* (Cully *et al.*, 1994; Dent *et al.*, 2000). In these studies CeGluClα3B formed homomeric channels capable of being activated by glutamate and IVM whereas the CeGluClβ subunit formed homomeric channels only able to respond to glutamate. GluClα3A did not respond to either ligand but it is unknown whether this was due the absence of ligand binding sites or an inability to form homomeric receptors. There is no direct orthologue of the *H. contortus* GluClα subunit in *C. elegans*. Based therefore on the properties of the

orthologous subunits of *C. elegans* and *H. contortus*, it was hypothesised that membranes expressing DiGluClα3B and not DiGluClα3A would bind [^{3}H]-IVM.

Delivery of recombinant DNA by transient transfection, which can be achieved using a variety of biochemical or physical methods, is a convenient and rapid way of obtaining high levels of protein expression, if only temporarily, in mammalian cells. The transfections detailed in this chapter were conducted using a calcium phosphate mediated approach, a well-established and inexpensive method for such transient expression. DNA and calcium phosphate form an insoluble precipitate, when mixed, and it is believed that this becomes attached to the cell's membrane where it is internalised by endocytosis (Sambrook & Russell, 2001). The mammalian cell-lines COS-7 and HEK-293T were chosen for these experiments as both are routinely for transient transfections. Indeed, COS-7 cells were used for expression of the individual *H. contortus* GluCl subunits, as described previously, although the DNA transfection procedure was mediated by lipofection rather than calcium phosphate (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002). Both COS-7 and HEK-293T cell-lines express high levels of the SV40 large tumour antigen (T), which initiates viral DNA replication at the SV40 origin, a region included in the expression vectors chosen for these experiments. Therefore high copy-numbers of these plasmids could be generated within the cells, theoretically enhancing the level of transcription (Spector *et al.*, 1998).

The initial strategy for these experiments was to conduct small-scale transfections, so that the conditions required for the expression of these subunits could be optimised. Larger-scale transfections were then to be carried out to ensure there was sufficient membrane protein available with which to perform the radioligand binding assays. For the small-scale procedure the DiGluClα3 cDNAs were cloned into specialised plasmids, which enabled the incorporation of an epitope-tag at the C-terminal of the expressed polypeptides. Following transfection, protein expression was monitored by immunofluorescence, using primary antibodies to the epitope-tags and secondary species-specific antibodies coupled to the fluorescent label, FITC. The larger scale transfections were to be carried out using mammalian expression plasmids that did not incorporate epitope tags, removing the possibility that the additional amino acid residues may alter the binding of the radioligand.

4.2 Results

4.2.1 Production of epitope-tagged GluClα3 plasmid constructs

The DiGluClα3 cDNAs for cloning into the C-terminal tag expression plasmids had to be amplified by PCR, using previously made clones as template DNA. The common sense oligonucleotide primer, SDG2/3TAG, was used in tandem with either with the anti-sense primer ASDG2TAG, to amplify the GluClα3A cDNA, or ASDG3TAG to amplify the GluClα3B cDNA; the nucleotide sequences for all primers detailed in this chapter are found in appendix A. Both PCRs used a simple 3-step program, with an annealing temperature of 50°C. Importantly, the anti-sense primers for each subunit were designed so that the stop codons of each cDNA were removed, therefore enabling the polypeptides to be translated in frame with the 3' tag. In addition, restriction sites were incorporated into all the primers to facilitate cloning. The amplified GluClα3A and GluClα3B cDNAs, both of the expected size of 1.3kb, were ligated into the C-terminal tag expression plasmids pFLAG-CMV-5a (at the PstI/BamHI sites) and pCMV/*myc*/cyto (at the PstI/XhoI sites) respectively. Transcription from both of these plasmids is under the control of the strong constitutive mammalian CMV (cytomegalovirus) promoter (see Stinski, 1999 for review) thus facilitating high-level protein expression. Recombinant clones were identified by restriction digestion using the aforementioned restriction enzymes; one of these clones was sent to be sequenced (see chapter 3 for sequencing primers). Fully sequencing the construct ensured firstly, that no nucleotide errors had arisen during PCR and secondly, that the subunit coding region was in-frame with the epitope-tag. Following sequencing, maxipreps were made for each construct to ensure sufficient DNA was available for the transfections.

Later experiments used similar plasmid constructs that differed only by the inclusion of the Kozak sequence, GCCACC, just prior to the start codons of both DiGluCl cDNAs. Kozak sequences affect the efficiency at which ribosomes initiate translation at a particular ATG. The sequence incorporated into these plasmids has been shown to produce maximum initiation efficiency, i.e. little translation occurs at other ATGs within the mRNA sequence, thus increasing protein expression (Kozak, 1986, 1999). Indeed, this Kozak sequence was used during the expression of HcGluClα subunits in COS-7 cells (Forrester *et al.*, 2002). The Kozak sequence was incorporated during PCR, being included in the newly designed

sense primer, SDG2/3TAGK, which aside from the six-nucleotide addition was identical to the original sense primer used. The anti-sense primers used in the PCRs were the same as those employed to amplify the original cDNAs (ASDG2TAG and ASDG3TAG). The PCR programs and subsequent cloning procedures were otherwise identical to those used to make the initial C-terminal tagged expression constructs. Figure 4.1 shows the products amplified by these PCRs, which were of the expected size of 1.3kb. Figure 4.2 shows restriction digests of the two Kozak-incorporated expression constructs. Following sequencing, maxipreps of each plasmid were made.

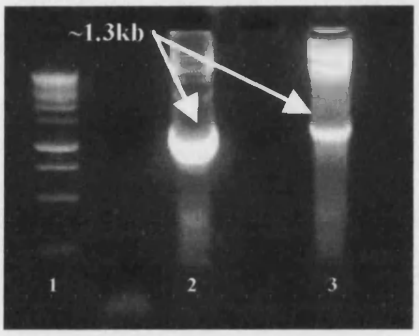


Figure 4.1. Amplification of GluClα3 cDNAs (including the Kozak sequences) for cloning into C-terminal expression vectors. Lane 1: 1kb DNA ladder; lane 2: GluClα3A PCR product; lane 3 GluClα3B PCR product; Both PCRs used a 3-step program, with an annealing temperature of 50°C. The primer pairs used to amplify the α3A and α3B subunit cDNAs were SDG2/3TAGK with either ASDG2TAG or ASDG3TAG respectively.

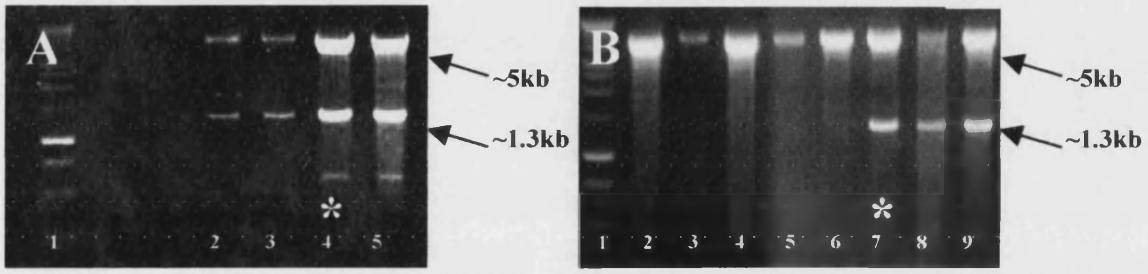


Figure 4.2. Restriction analysis of (Kozak sequence including) GluClα3A:pFLAG-CMV-5a (A) and GluClα3B:pCMV/*myc*/cyto (B) transformants. A: lane 1: 1kb DNA ladder; lanes 2-5: GluClα3A:pFLAG-CMV-5a transformants digested with PstI/BamHI. **B:** lane 1: 1kb DNA ladder; lanes 2-9:GluClα3B:pCMV/*myc*/cyto transformants digested with PstI/XhoI. Recombinant plasmids for both constructs were characterised by the presence of one band at ~5kb, the linearised vector, and one band at ~1.3kb, the insert cDNA, as indicated by the arrows. The asterisks denote the clones sequenced and used in the subsequent transformations.

Maxipreps were also made for the plasmid constructs used in the control transformations. A GFP expressing plasmid construct (GFP-CD36; obtained from V. Portillo, University of Bath) was used to initially assess the efficiency of transfection protocol. To assess the

abilities of each C-terminal epitope-tag to be detected, plasmid constructs expressing HcGluClβ-FLAG (obtained from V. Portillio) and hTRPC3-myc (obtained from I. Franklin, University of Bath) were used. hTRPC3 (Human Transient Receptor Potential type C) is a type of calcium channel. Importantly, the C-terminal tag expression vectors used to make these constructs (pFLAG-CMV-5a and pCMV/*myc*/cyto) were identical to those used to express the DiGluClα3 subunits.

4.2.2 Expression of epitope-tagged DiGluClα3 subunits

As outlined in the introduction to this section, calcium phosphate mediated transient transfection was used to deliver the plasmid DNA into the COS-7 and HEK-293T mammalian cell-lines. Expression of GFP was initially used as the marker for optimising the transfection conditions. The maximum transfection efficiency observed, 20-30%, was obtained following addition of 5-7μg plasmid DNA using the protocol outlined in the methods section. These conditions appeared to be optimal for both cell-lines. Factors altered during the optimisation of the transfection procedure included the length of time the DNA-calcium phosphate mixture was incubated with the cells (either 5-6 hours or overnight as indicated by different methodologies), the subjection or not of the cells to a "glycerol shock" (see method section 2.2.3.2.3) following removal of the mixture, and the length of time (24 to 48 hours) allowed for protein expression before cell fixation. Results from transfection experiments using the positive control constructs (i.e. HcGluClα3-FLAG and TRP3-myc) indicated that similar conditions also lead to optimal transfection efficiencies and expression of these proteins. Again, both cell-lines exhibited similar transfection efficiencies under similar experimental conditions, although these were generally lower than those observed for GFP.

Unfortunately, over a range of 1-10μg DNA the epitope-tagged DiGluClα3 subunits, using either expression construct (i.e. with or without the Kozak sequence) appeared to be transfected with a very poor efficiency in both cell-lines. Expression of the GluClα3A-FLAG subunit could occasionally be detected in both cell-lines although only at the rate of 1-5 cells per coverslip. Typically however no expression was observed. No expression was detected for the DiGluClα3-myc subunit in either cell-line, using either construct. Altering the protocol in ways like those described above for the initial "optimisation" of this

transfection method did little to enhance the transfection efficiency or expression of the tagged DiGluClα3B subunits in either cell-line. Figure 4.3 summarises the typical results following transfection of the subunit and control DNA into COS-7 cells. The results however were also indicative of the expression observed in HEK-293T cells as well.

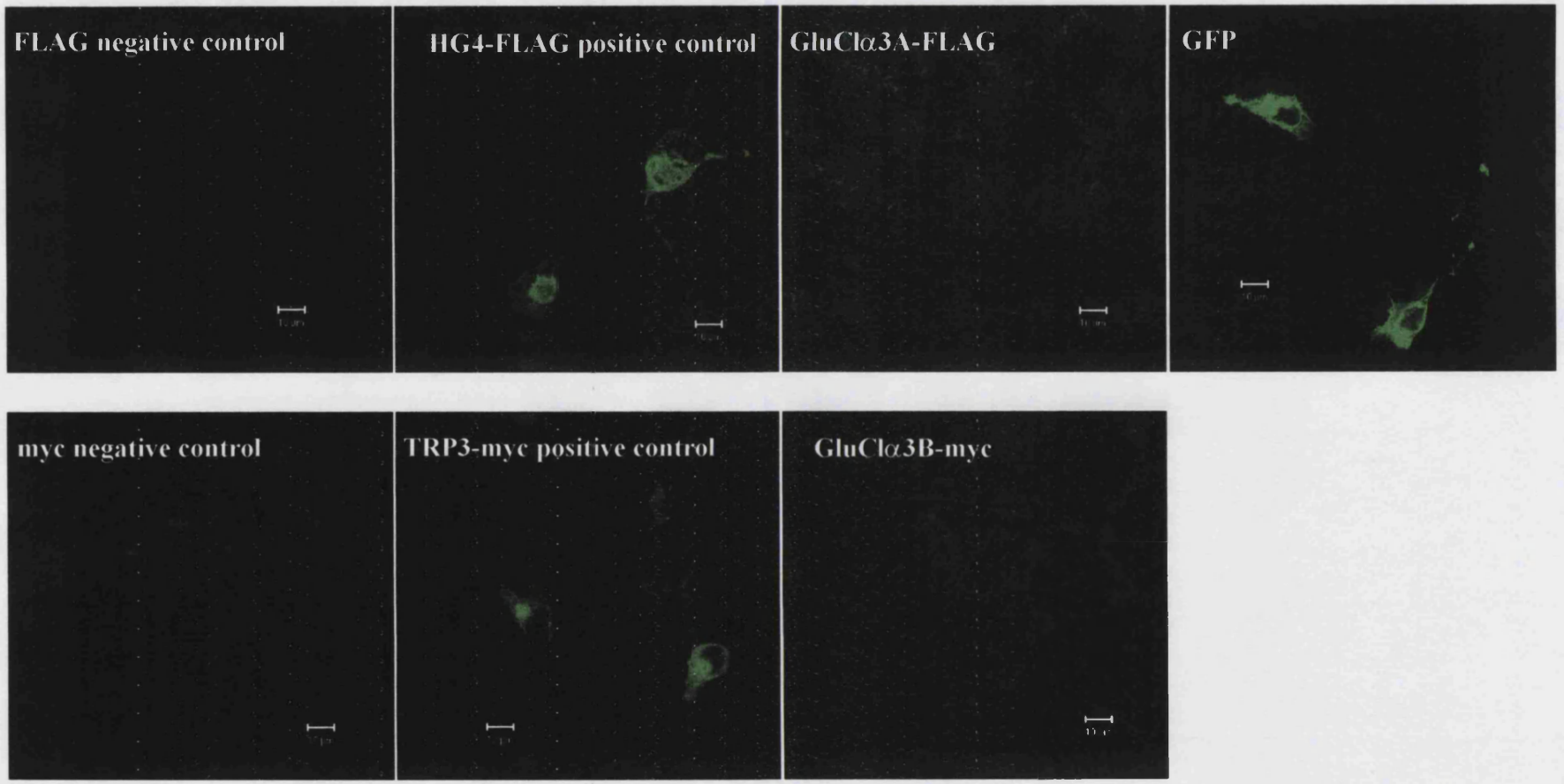


Figure 4.3. Expression of the epitope-tagged DiGluClα3 subunits in COS-7 cells. Forty-eight hours following transfection the cells were fixed. They were subsequently incubated (except the GFP control) with primary antibody (either mouse anti-FLAG, 1/1500 dilution or mouse anti-myc, 1/200 dilution) and secondary antibody (FITC-conjugated anti-mouse, 1/200 dilution) before being viewed under a confocal microscope. Negative controls were included for both subunits, where staining was conducted on untransfected cells. Antigen detection was typically only observed in the positive controls for both epitope-tags.

4.3 Discussion

The epitope–tagged DiGluClα3 subunits were expressed poorly (i.e. few cells expressed the polypeptides) in COS-7 and HEK-293T cell-lines, preventing the proposed radioligand binding assays from being performed. Expression of the DiGluClα3A-FLAG subunit could occasionally be detected in both cell-lines but only at the rate of 1-5 cells per coverslip. The levels of expression within these cells appeared to be quite high according with their brightness, although was never quantified. Usually however, no expression of this subunit could be detected. Expression of the DiGluClα3B-myc subunit was not detected in either cell-line. Discussed below are possible explanations for the poor expression observed. Additionally, alterations to the procedures used that may lead to improved transfection efficiencies and protein expression are considered.

There are a number of empirical measures that are often taken in attempting to improve transfection efficiencies and protein expression. As alluded to earlier, there are a number of biochemical and physical methods for transiently transfecting DNA into mammalian cells, which could be used instead of the calcium phosphate approach undertaken here. A commonly used alternative is lipofection-mediated transfection, which has been previously used to successfully express selected *H. contortus* GluCls in COS-7 cells (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002). Further studies, interestingly, showed that calcium phosphate mediated transfection was actually sufficient for expressing epitope-tagged HcGluClα and HcGluClβ subunits in COS-7 cells (V. Portillo, pers. comm.). This may indicate that these proteins are simply more amenable to expression in mammalian cells than the *D. immitis* GluCls, a topic briefly considered below. In addition to the transfection methodology the expression system may also be changed, often to an alternative mammalian cell-line or indeed to cell-lines from organisms more closely related to the species of interest. For example, the *Drosophila* S2 cell-line has been used to express selected *H. contortus* GluCl subunits. For the epitope-tagged HcGluClα subunit the transfection efficiency achieved using these cells appeared to be significantly greater than that achieved in COS-7 cells, validating their use (V. Portillo, pers. comm.).

In contrast to the empirical changes proposed above, a more careful examination of the transfection procedure used here indicates further alternative measures that could be

implemented to improve protein expression. The success of the positive controls, in terms of both efficiency and levels of protein, suggest that the major problems associated with expression of the GluClα3 subunits probably occurred after transcription, either at the level of translation or post-translation. This reasoning derives from the fact that prior to translation the regulation of the combined processes of DNA uptake, replication of plasmid DNA and transcription, was largely dependent on common nucleotide sequences found within the expression vectors and not the insert cDNA. For example, the CMV promoter was the regulatory sequence that principally governed transcription. A simple way to test this hypothesis would be to perform RT-PCR on transfected cells, to see if the GluClα3 mRNAs could be amplified. Presence of such transcripts would evidently support the deductions made above.

It is interesting to note that potentially the GluClα3-tagged subunits were expressed in both cell-lines with acceptable efficiencies and that simply the levels of expression within the cells were below the sensitivity of the immunofluorescence method used to detect them. The use of Western Blotting may resolve whether both subunits were consistently expressed. If either subunit could be detected using this method it may support the hypothesis discussed above. However, the establishment of low-level expression would still not be sufficient to begin radioligand binding assays as the number cells required to generate enough protein for such experiments would be unfeasible.

One possibility for explaining the poor expression of the DiGluCl subunits in mammalian cells, at the level of translation, is the asymmetrical use of codons in different species. Filarial nematodes have a pronounced adenine-thymine (AT) bias of ~70% within their DNA, which extends to both coding and non-coding regions (Rothstein *et al.*, 1988; Fadiel *et al.*, 2001). In contrast the situation in mammals is overall more balanced with a ~50% AT content to their DNA. Within the codons of *D. immitis* the AT bias is most clearly evident at the third or wobble position (Fadiel *et al.*, 2001). Therefore the possibility exists that high level expression of the DiGluClα3 subunits may have been hindered by frequent use, within the coding cDNAs, of what in mammalian cells may be considered unusual or rare codons. A recent study by Slimko and Lester (2003) highlighted this issue of asymmetric codon use between species. Their objective was to express the *C. elegans*

GluClα1 and GluClβ genes in rat hippocampal neurons. Expression however was poor so they constructed synthetic genes for each subunit using codons that were optimal for mammalian cells. The optimisation process provided a 6- to 9-fold increase in expression of these subunits. Interesting, the coding regions of *C. elegans* DNA have a similar base composition (i.e. ~50% AT) to mammalian DNA although a clear difference in codon usage exists (Emmons, 1988). As the *D. immitis* cDNAs possess a more exaggerated nucleotide bias, a potentially more striking improvement in expression following codon optimisation for these proteins may be predicted.

Alternatively, the poor expression observed maybe due to reasons associated with post-translational processes occurring within the endoplasmic reticulum (ER), the intracellular location where the majority of membrane proteins are folded and oligomerised (Hurtley & Helenius, 1989). For example, selected mammalian $GABA_A$ subunits (α1, β2, γ2) when expressed alone, or in double combinations (either α1γ2 or β2γ2) not known to be representative of the native receptors, are largely retained in the ER and rapidly degraded (Connolly *et al.*, 1996; Gorrie *et al.*, 1997). It is possible that a similar fate awaited the DiGluClα3 subunits when expressed alone, the continual rapid degradation preventing the protein build up that could have been detected by immunofluorescence. Co-expression with other GluCl subunits may therefore be required to stabilise expression however, which subunits should the DiGluClα3 polypeptides be co-expressed with? Due to the lack of *D. immitis* GluCl cDNAs the most obvious experiment may be to co-express both alternatively spliced forms of GluClα3. Alternatively, each subunit could be expressed with GluCl subunits from other nematode species on the basis that orthologous subunits may exist in *D. immitis*. Like any experiments of this type any subsequent binding data would be difficult to interpret, particularly from cross species subunit combinations, without complimentary studies supporting the potential association of the subunits *in vivo*. Finally, it remains possible that the addition of the epitope-tag may have affected the ability of the subunits to fold correctly, and hence assemble into viable receptors. They may therefore have been rapidly degraded preventing expression being detected.

To summarise, the major objective of this part of the study was to establish whether either DiGluClα3 subunit was a molecular target of AMs. Unfortunately, due to poor expression

of these subunits in the selected mammalian cell-lines, the proposed radioligand binding assays that would have addressed this issue were unable to be performed. Hopefully the measures considered above, may be of some use in directing future transient expression of *D. immitis* GluCl subunits.

Chapter 5

Pharmacological Study of GluClα3 Subunits using Two-Electrode Voltage Clamping

5.1 Introduction

Following the difficulties encountered expressing the DiGluClα3 subunits in mammalian cell-lines an alternative approach was sought to investigate their pharmacology. The method chosen to achieve this objective was that of two-electrode voltage clamping (TEVC) using *Xenopus laevis* (the South African clawed frog) oocytes as the surrogate expression system for the recombinant ion channels. The TEVC method uses one electrode to monitor the transmembrane potential of the oocyte (relative to a bath electrode), which is compared to the desired holding or clamping potential set by an amplifier (often -80mV). Current is then injected into the oocyte via the second electrode to minimise any difference observed. It is the change in current, which is measured during a recording, as the amplifier attempts to maintain the desired holding potential in the face of ion flow through activated channels following, in this case, ligand application (Betrand *et al.*, 1991).

Aside from the polypeptide encoded by *glc-4*, all other *C. elegans* GluCl subunits have been studied (to varying degrees) using this methodology. The GluClα2A, α2B, α3B, and α4 subunits are all capable of forming homomeric channels in oocytes that are gated by glutamate and IVM (Dent *et al.*, 1997; Vassilatis *et al.*, 1997b; Dent *et al.*, 2000; Horoszok *et al.*, 2001). GluClβ forms homomeric channels gated by glutamate but not IVM where as reverse is true for GluClα1; the GluClα1β and GluClα2Bβ heteromers are gated by both ligands (Cully *et al.*, 1994; Vassilatis *et al.*, 1997b). Only GluClα3A appears unable to form homomeric receptors activated by either ligand (Dent *et al.*, 2000). The only other nematode GluCl subunit to have been studied using this method is the HcGluClα, which was recently shown to form homomeric channels gated by both ligands (Forrester *et al.*, 2003).

As observed in these studies, both glutamate and IVM induce highly characteristic current in oocytes expressing sensitive GluCls. Glutamate elicits fast-onset chloride currents that are fully reversible upon removal of the ligand; the channels exhibit varying degrees of desensitisation depending on their constituent subunits and concentration of glutamate used. IVM however, induces slow-onset chloride currents that do not desensitise and are essentially irreversible despite removal of the ligand and prolonged washing with buffer

(Cully *et al.*, 1994; Dent *et al.*, 1997, 2000; Vassilatis *et al.*, 1997b; Horoszok *et al.*, 2001; Forrester *et al.*, 2003); the irreversible nature of the currents is partially explained by the slow dissociation of IVM from its binding site (Schaeffer & Haines, 1989). The EC_{50} values generated for glutamate in these studies (including both the homomeric and heteromeric GluCls) range from 8.4μM to 1.9mM (Cully *et al.*, 1994; Dent *et al.*, 1997; Vassilatis *et al.*, 1997b; Horoszok *et al.*, 2001; Forrester *et al.*, 2003). These figures are generally consistent with glutamate concentrations shown to gate chloride currents in nematode tissue preparations (Martin, 1996; Pemberton *et al.*, 2001). Gating by IVM of sensitive GluCls in oocytes generally requires concentrations of ligand ≥10nM; the determined EC_{50} values range from 103nM to 400nM (Cully *et al.*, 1994; Dent *et al.*, 1997, 2000; Vassilatis *et al.*, 1997b; Horoszok *et al.*, 2001; Forrester *et al.*, 2003). These figures are significantly higher than the concentrations of IVM that have been shown to bind individual *H. contortus* GluCl subunits (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002) and the concentrations able to paralyse pharyngeal pumping and motility in *H. contortus, C. elegans* and selected other nematodes *in vitro* (Kass *et al.*, 1980; Avery & Horvitz, 1990; Gill *et al.*, 1991, 1995; Geary *et al.*, 1993; Arena *et al.*, 1995; Paiement *et al.*, 1999). Interestingly, IVM at lower concentrations (<10nM) potentiates currents induced by sub-maximal concentrations of glutamate in oocytes expressing the GluClα1β heteromers (Cully *et al.*, 1994).

The overall objective for these experiments was identical to that outlined in the last chapter, i.e. to express the DiGluClα3 subunits in a system that would allow their pharmacology to be investigated. Based on the qualitative responses of their *C. elegans* orthologues, it was hypothesised that the DiGluClα3B subunit would form homomeric receptors in oocytes that would be gated by glutamate and IVM. Conversely, oocytes injected with DiGluClα3A cRNA were hypothesised not to respond to either ligand. Any responses obtained, whether accepting or rejecting these hypotheses, would direct further experiments. In tandem with these studies it was hoped that glutamate and IVM dose response curves could be generated for the CeGluClα3B homomers and in addition, to establish whether the CeGluClα3 subunits could associate with each other to form heteromeric channels.

Xenopus oocytes have been extensively used to characterise neurotransmitter receptors and other recombinant ion channels, as they are highly amenable to electrophysiological methods such as TEVC. The late stage oocytes (V and VI) typically used for these experiments reach 1-1.2mm in diameter, the large size facilitating both microinjection of the mRNA into the cytoplasm (or in some cases nuclear injection of cDNA) and the subsequent insertion of the microelectrodes. In addition, oocytes readily synthesise exogenous proteins when injected with foreign mRNA; post-translational modifications, assembly, sorting and other processes all appear to proceed appropriately within the cells. Finally, although they do possess some voltage-operated ion channels and G-protein coupled receptors these usually offer little interference to studying recombinant ion channels. With particular reference to this study, no electrical responses are observed from oocytes following application of glutamate. There are however, some disadvantages to this system, not least the short duration of the expression period (maximum of two weeks) compared to stably transfected cell-lines. Also, few cells can be assessed during one experiment and oocyte reliability is often seasonally dependent, with cells used outside this period liable to exhibiting erratic non-functional responses (Sigel, 1990).

5.2 Results

5.2.1 Preparation of *in vitro* transcribed capped GluClα3 RNA

As the transcription kit used a T7 RNA polymerase to generate the capped GluClα3 RNAs, each subunit cDNA had to be orientated in the 5'-3' direction downstream of a T7 promoter. Initially, each subunit cDNA was sub-cloned from existing constructs into plasmids incorporating this region. DiGluClα3A and α3B were sub-cloned from pCR®-Blunt II-TOPO constructs into pcDNA3 (KpnI/XbaI sites) and pcDNA3.1- (EcoRV/BamHI sites) respectively. CeGluClα3A and α3B were sub-cloned from pBluescript constructs (obtained from A. J. Wolstenholme) into pcDNA3.1- (XbaI/KpnI sites) and pcDNA3 (NotI/XbaI sites) respectively. The new constructs were linearised, using the 3' restriction site and cRNA was transcribed as outlined in section 2.2.4.1. cRNA was also transcribed from a CeGluClα4/pGEM®-T Easy construct (obtained from L. Horoszok), linearised at the 3' end with SalI, to be used as the positive control.

Application of glutamate to *Xenopus* oocytes injected with CeGluClα4 cRNA elicited the typical robust inward currents previously described (Horoszok *et al.*, 2001). However, no responses to glutamate were observed with oocytes injected with cRNA for any of the four GluClα3 subunits ($n \geq 10$ tested per subunit per frog; oocytes were tested from at least four frogs). These recordings were made over a period of 1-4 days post-injection and used concentrations of 1-10mM glutamate to elicit a response. As CeGluClα3B had been previously shown to form homomeric channels capable of responding to glutamate (Dent *et al.*, 2000), it was concluded that this subunit, at least, should show similar properties in these experiments. Therefore, subsequent experiments (those considered in the results section) used subunit cRNAs that included the 5' untranslated leader sequence from the coat protein mRNA of alfalfa mosaic virus (referred to the 5'-AMV sequence in the text) directly upstream of the start codons. The 5'-AMV sequence has been used, as one of several methods, to increase the translational efficiency of mRNA in *Xenopus* oocytes (Jobling & Gehrke, 1987; Figl *et al.*, 1998).

The 36-nucleotide 5'-AMV sequence was incorporated by PCR, being included in the sense primer for each subunit (see appendix A for primer details). The DiGluClα3A and α3B 5'-AMV-incorporated cDNAs were amplified by PCR using the common sense primer,

SDiGluClα3AMV, with either the anti-sense primer ASDiGluClα3AAMV or ASDiGluClα3BAMV respectively. The CeGluClα3A and α3B 5'-AMV-incorporated cDNAs were amplified by PCR using the common sense primer, SCeGluClα3AMV, with either the anti-sense primer ASCeGluClα3AAMV or ASCeGluClα3BAMV respectively. All four PCRs used a conventional 3-step program with an annealing temperature of 50°C. The amplified cDNAs were all cloned into pcDNA3.1- at XbaI/NotI sites, ensuring they were all correctly orientated 5'-3' downstream of a T7 promoter (figure 5.1); the restriction sites were included in the primers. These constructs were subsequently linearised as before at the 3' restriction site, i.e. NotI, and were used as the template DNA in the cRNA transcription reactions (figure 5.2).

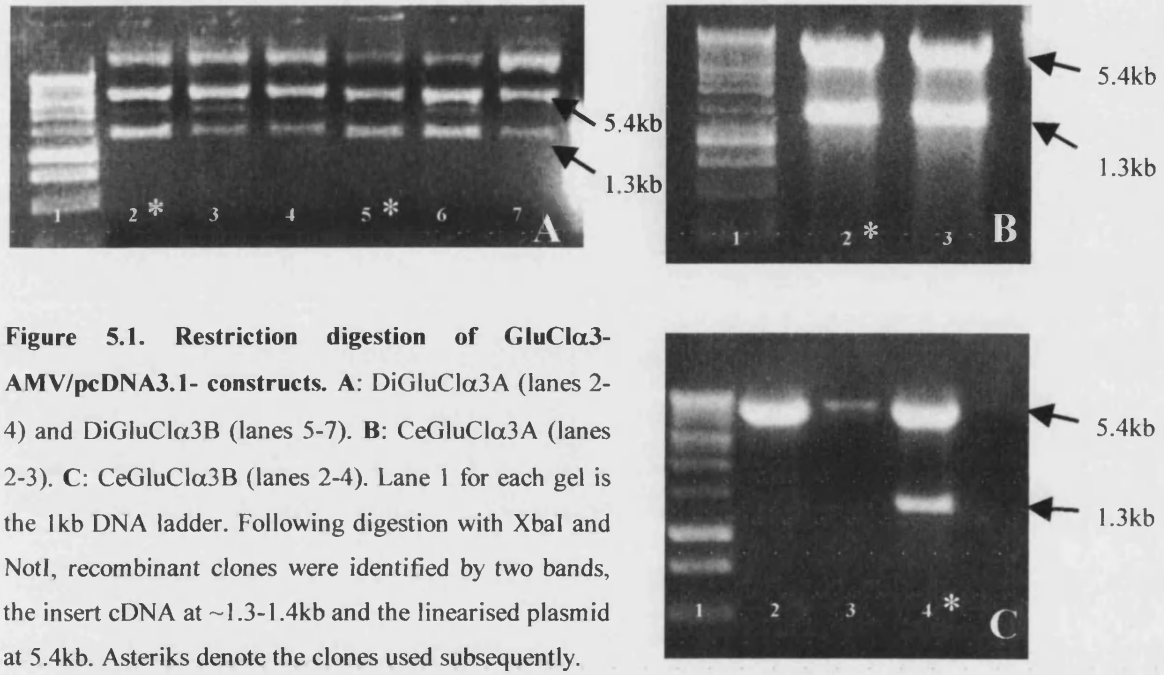


Figure 5.1. Restriction digestion of GluClα3-AMV/pcDNA3.1- constructs. **A**: DiGluClα3A (lanes 2-4) and DiGluClα3B (lanes 5-7). **B**: CeGluClα3A (lanes 2-3). **C**: CeGluClα3B (lanes 2-4). Lane 1 for each gel is the 1kb DNA ladder. Following digestion with XbaI and NotI, recombinant clones were identified by two bands, the insert cDNA at ~1.3-1.4kb and the linearised plasmid at 5.4kb. Asteriks denote the clones used subsequently.

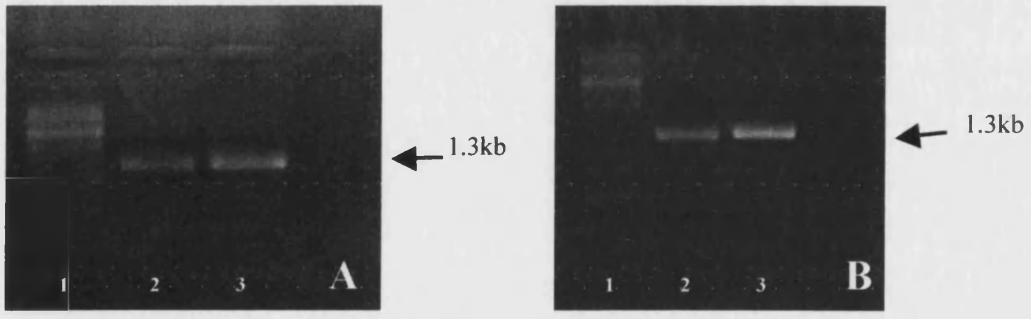


Figure 5.2. *In vitro* transcribed cRNA for the GluClα3 subunits of (A) *D. immitis* and (B) *C. elegans*. For each gel, lane 1: RNA ladder. Lanes 2 and 3: cRNA samples for GluClα3A and GluClα3B subunits respectively.

5.2.2 Actions of glutamate and IVM on oocytes injected with DiGluClα3 cRNAs

Xenopus oocytes injected with DiGluClα3B cRNA were shown to respond to glutamate in the characteristic manner when clamped at a holding potential of –80mV: the agonist elicited a dose-dependent, rapid-onset, inward current that was fully reversible. These channels also exhibited rapid desensitisation (figure 5.3A). Unfortunately and despite extensive screening (≥10 cells tested per frog; at least four animals tested in total), only a limited number of oocytes responded to glutamate (n=7) and of these only three exhibited maximum currents >30nA. This made generation of a glutamate dose response curve for DiGluClα3B homomers unfeasible. Instead, two of these glutamate-sensitive oocytes were used to demonstrate that DiGluClα3B homomers are also gated by 1μM IVM (figure 5.3B). Characteristically, the current elicited by IVM had a slow onset, did not desensitise, and was essentially irreversible despite removal of the ligand and washing for 5 minutes with ND98 buffer only. No currents were observed upon application of glutamate (10-100mM) to oocytes (n≥10 tested per frog; four animals tested in total) injected with DiGluClα3A cRNA. In addition, no responses were observed upon application of glutamate or IVM at concentrations of 10mM or 1μm respectively to the uninjected or ddH_2O injected oocytes (n=5 for both).

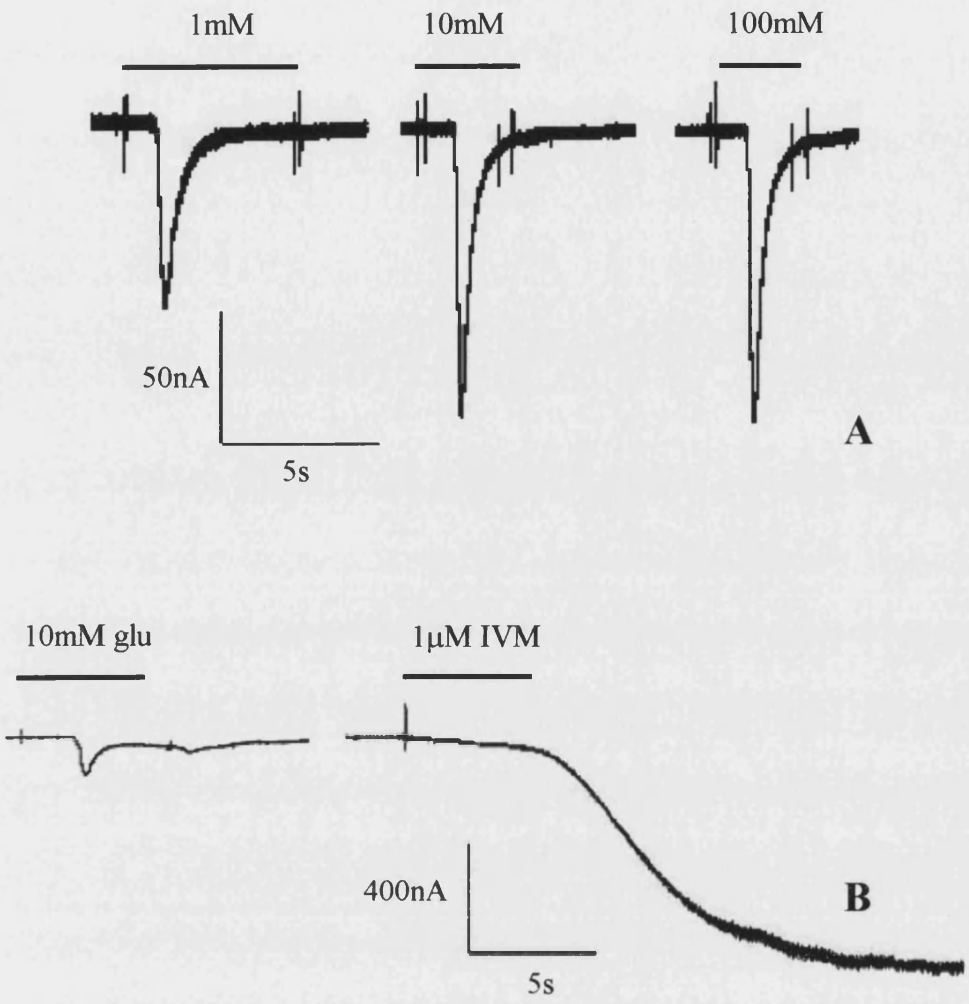


Figure 5.3. Glutamate-gated and IVM-gated currents in oocytes injected with *D.immitis* GluClα3B cRNA and voltage-clamped at –80mV. For all traces the downward deflections denote the inward nature of the currents. The horizontal bars indicate the period of ligand application, the numbers above indicating the concentrations used. **A:** Example traces of the dose-dependent glutamate-gated currents. The currents generated by glutamate exhibited both a rapid-onset and a rapid desensitisation, and were fully reversible upon removal of the ligand. **B:** Example traces of the glutamate-gated and IVM-gated currents generated from the same oocyte injected with DiGluClα3B cRNA. Unlike the glutamate-gated currents, IVM induced a slow-onset, essentially irreversible current, which exhibited no desensitisation.

5.2.3 Quantitation of the glutamate EC_{50} for CeGluClα3B homomers

Xenopus oocytes injected with *in vitro* transcribed CeGluClα3B cRNA and clamped at a holding potential of -80mV, exhibited robust inward-currents that rapidly desensitised in response to glutamate (figure 5A). In contrast, the CeGluClα3A subunits appeared to be unable to form glutamate sensitive channels in oocytes; the recordings were made on ≥10 oocytes per frog (four animals in total), using 10mM glutamate. These findings confirmed the observations made previously, as to the qualitative response of each subunit to glutamate when expressed in this system (Dent *et al.*, 2000). As shown in figures 5A and 5B further investigation using oocytes injected with CeGluClα3B cRNA revealed that the dose dependent currents elicited by glutamate generated an EC_{50} value of 2.2 ± 0.12mM with responses peaking (i.e. the maximum response) at concentrations of ≥50mM. The Hill co-efficient of 0.72 ± 0.08 suggested only one molecule was required to gate the channel. Control oocytes (uninjected and injected with 50nl ddH_2O) failed to respond to glutamate at concentrations of 1, 10 and 100mM (n=5). Expression of this subunit in oocytes was almost exclusively found on days 2 and 3 post-injection. Although more consistent than was observed for the DiGluClα3B subunit, expression problems were also encountered for this polypeptide, preventing further experiments from being conducted.

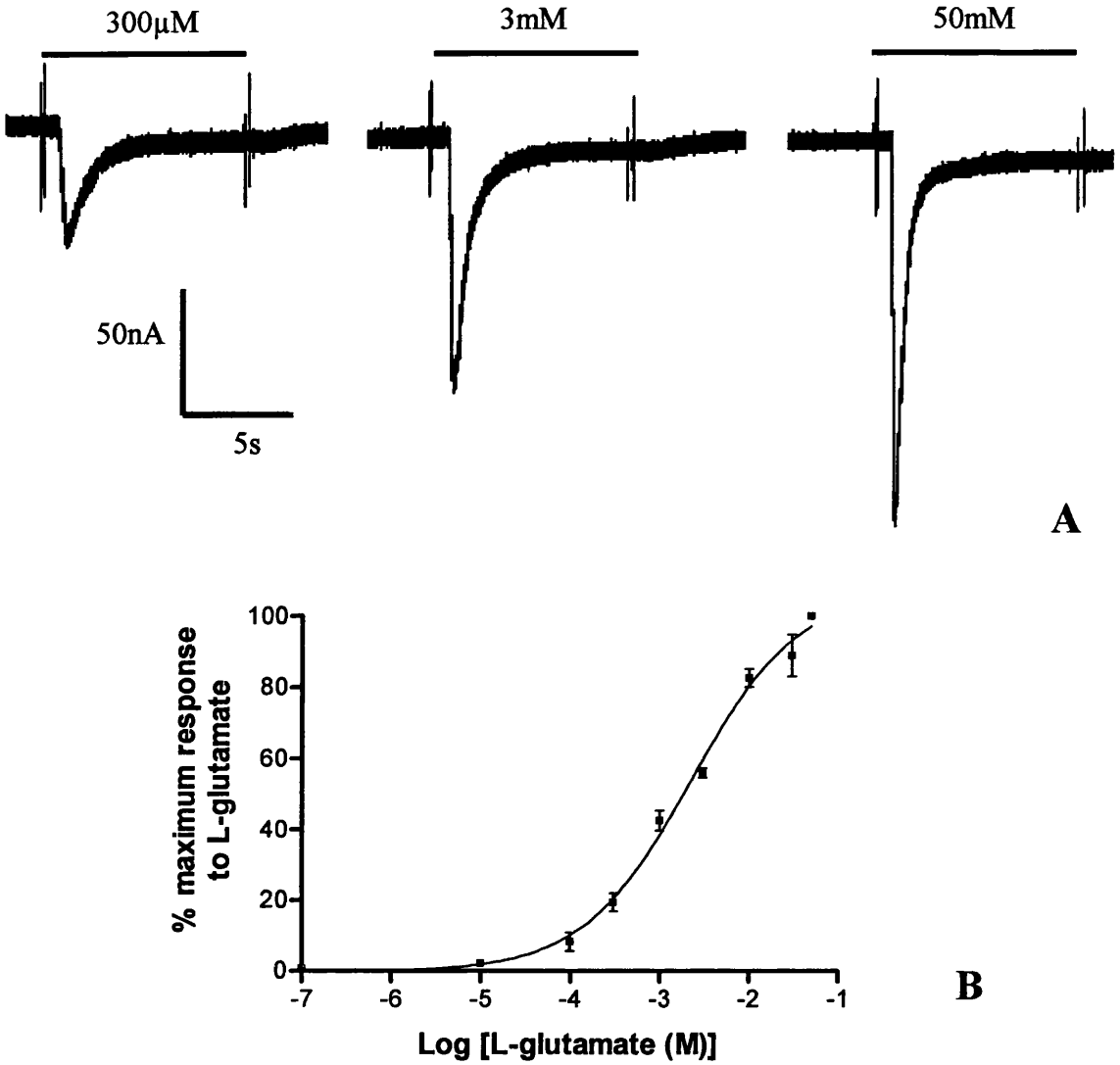


Figure 5.2 Dose dependent glutamate-gated currents in *Xenopus* oocytes injected with *C. elegans* GluClα3B cRNA and voltage clamped at –80mV. A: Example traces of the glutamate–gated currents; the downward deflections denote the inward nature of the currents. Horizontal bars relate to the duration of glutamate application, the numbers above indicating the concentrations used. The currents generated by glutamate exhibited both a rapid-onset and a rapid desensitisation, and were fully reversible upon removal of the agonist. **B:** Glutamate concentration response curve for oocytes injected with CeGluClα3B cRNA. Data were normalised to the maximum glutamate responses seen with each oocyte and are shown as the standard error of the mean of five oocytes (taken from two frogs). The observed currents generated an EC_{50} value of 2.2 ± 0.12mM and a Hill coefficient of 0.72 ± 0.08. Uninjected and ddH_2O injected controls did not exhibit any response to glutamate.

5.3 Discussion

As hypothesised, DiGluClα3B subunits are able to form homomeric channels in *Xenopus* oocytes that are gated, in typical fashions, by both glutamate and IVM. In addition, initial results indicate that DiGluClα3A subunits cannot form homomers sensitive to glutamate. These data are in general agreement with those responses observed for their *C. elegans* orthologues (Dent *et al.*, 2000); the effect of IVM on oocytes injected with DiGluClα3A subunit cRNA remains untested. The ability of IVM to activate both *C. elegans* and *D. immitis* GluClα3B homomers complements a study that demonstrated that HcGluClα3B bound [^{3}H]-IVM with high affinity (K_D=70pM) (Cheeseman *et al.*, 2001). HcGluClα3A did not bind [^{3}H]-IVM (Cheeseman *et al.*, 2001), which is also in line with the inability of CeGluClα3A subunits to form IVM-sensitive homomers in oocytes (Dent *et al.*, 2000; this chapter). Given this apparent conservation in, albeit limited, pharmacology across species for the GluClα3 subunits it would be predicted that the DiGluClα3A subunits were also unable to form IVM-sensitive homomeric channels. This perhaps should not be surprising given the high amino acid sequence identity between orthologous GluClα3 subunits (≥80%).

With regard to the results from the application of glutamate, it remains unknown whether the GluClα3A subunits of both *D. immitis* and *C. elegans* are deficient in agonist binding, coupling binding to gating or indeed the ability to associate into homomeric channels. The pattern of alternative splicing of the *avr-14* gene, conserved across the species examined, results in the GluClα3A and α3B subunits having identical N-terminals. These regions are presumed to contain all the elements of glutamate binding, indicating the first of these possibilities is unlikely (see chapter 3; Laughton *et al.*, 1997a; Jagannathan *et al.*, 1999). Both alternative explanations remain plausible, as illustrated in earlier chapters for other LGICs: CeGluClα1 homomers are unable to couple glutamate binding to channel gating (Etter *et al.*, 1996) where as selected mammalian $GABA_A$ subunits (α1, β2, γ2) will only form functional receptors *in vitro* that are equivalent in subunit composition to the native channels *in vivo* (Connolly *et al.*, 1996; Gorrie *et al.*, 1997).

All three reasons given above could also explain why CeGluClα3A subunits are unable to form IVM-sensitive homomers in oocytes (Dent *et al.*, 2000); for glutamate-binding site read IVM binding site, as it appears unlikely these are one and the same (discussed in chapter 6). However, if we assume that the pharmacology of these subunits is conserved across species then it appears unlikely that an inability to couple binding to gating alone, explains the lack of IVM response because as stated, HcGluClα3A subunits cannot bind [^{3}H]-IVM (Cheeseman *et al.*, 2001). Actually, given this assumption, there are only two likely explanations for the observed pharmacology of GluClα3A subunits in oocytes. Either they simply cannot associate into homomeric channels, regardless of other factors, or they form homomers that do not possess an IVM binding site and are deficient in coupling glutamate binding to gating. Either way, both scenarios would suggest that GluClα3A subunits do not form homomeric channels *in vivo*.

The glutamate dose response curve for CeGluClα3B homomers generated an EC_{50} value of 2.2 ± 0.12mM, which is comparable to those described for the *C. elegans* GluClα2A (2mM) (Dent *et al.*, 1997) and GluClα4 (1.9mM) homomers (Horoszok *et al.*, 2001). All these figures are significantly higher than EC_{50} values determined for oocytes injected with *C. elegans* mRNA (EC_{50}=300μM) (Arena *et al.*, 1992) and a GluCl described in a *C. elegans* pharyngeal preparation (EC_{50}=166μM) (Pemberton *et al.*, 2001). This suggests CeGluClα3B subunits do not form homomeric receptors *in vivo*. Interestingly, the Hill coefficient for CeGluClα3B was only 0.72 ± 0.08, suggesting only one glutamate molecule was required to gate the channel. Hill coefficients determined for other GluCls (see table 1.2) have generally indicated that more than one molecule is required to gate the channel, lending support to the notion that this subunit does not form homomers *in vivo* (Arena *et al.*, 1992; Cully *et al.*, 1994; Cully *et al.*, 1996a; Vassilatis *et al.*, 1997a; Dent *et al.*, 1997; Horoszok *et al.*, 2001; Pemberton *et al.*, 2001; Forrester *et al.*, 2003). It remains a possibility however that due to the rapid desensitisation of the GluClα3B homomers in the presence of agonist, the maximum glutamate current was underestimated, which in turn may have subsequently lead to an underestimation of the Hill coefficient.

There is a great deal of scope for future work with the GluClα3 subunits from both *D. immitis* and *C. elegans*. The most obvious experiments for the DiGluClα3B homomers would be to quantify the glutamate and IVM responses. Additional generation of an IVM dose response curve for the CeGluClα3B homomers would permit the first direct comparison of quantitative pharmacology for orthologous GluCls. For the GluClα3B homomers of both species the subsequent experiments should probably be directed at confirming their GluCl status; the standard application of other common neurotransmitters and the determination of the reversal potential of the channels would suitably achieve this objective. As for the DiGluClα3A subunit, it needs to be established whether or not oocytes injected with this polypeptide's cRNA can respond to IVM or not. This would support (or not) the assumption that orthologous GluClα3 subunits exhibit conserved pharmacology.

Beyond those initial experiments two avenues of investigation may prove very revealing. Firstly, it would be very interesting to establish if low concentrations of IVM (<10nM) were able to potentiate sub-maximal applications of glutamate when applied to GluClα3B homomers from either species, as was demonstrated for CeGluClα1β heteromers (Cully *et al.*, 1994). The background for these experiments, as alluded to earlier, is that there is a clear discrepancy between the concentrations of AMs that activate recombinant channels in oocytes (>10nM; EC_{50} values range from 90-400nM) (Arena *et al.*, 1992; Cully *et al.*, 1994; Cully *et al.*, 1996a; Dent *et al.*, 1997; Vassilatis *et al.*, 1997a; Horoszok *et al.*, 2001; Forrester *et al.*, 2003) compared to those, which have been shown to bind individual GluCls (pM range) (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002) and exert their effects (paralysis of pharyngeal pumping and motility) on worms *in vitro* (≥0.1nM) (Kass *et al.*, 1980; Avery & Horvitz, 1990; Gill *et al.*, 1991, 1995; Geary *et al.*, 1993; Arena *et al.*, 1995; Paiement *et al.*, 1999). This has lead to some debate regarding the true mechanism of action of the AMs at low concentrations (<10nM) (Pemberton *et al.*, 2001; Yates *et al.*, in press), a subject which is expanded upon in chapter 6.

Due to the pharmacology outlined above, it appears unlikely the CeGluClα3A subunits form homomeric receptors *in vivo*. By inference, partially supported by the limited data available, the same may well be true for the *D. immitis* orthologues. Therefore, a second interesting avenue of investigation may be to conduct TEVC recordings on oocytes injected

with multiple GluCl subunit cRNAs to see if they able form heteromeric channels; these may (or may not) be more indicative of the *in vivo* receptors. In *C. elegans*, limited subunit combinations (GluClα1β and GluClα2Bβ) have already been examined (Cully *et al.*, 1994; Vassilatis *et al.*, 1997b). As the CeGluClα3 subunits appear to be widely expressed in the nervous system, it would not be surprising if they were be able to associate with a number of other GluCl subunits to form heteromeric receptors, including with each other (Dent *et al.*, 2000). Elucidating the correct subunit stoichiometries of the native receptors will be of major importance. For example, it may explain the different sensitivities of the nematode's biological targets, i.e. pharyngeal pumping, motility and fecundity, to AM action. Activation of native receptors may also require lower concentrations of AM than observed for the GluCls so far expressed in oocytes, dispelling the debate surrounding the mechanism of action of these drugs (see chapter 6). Finally, learning the true nature of the *in vivo* channels would provide a much more accurate target for screening further potential anthelmintics. Obviously, these experiments are certainly possible in *C. elegans*, as it appears likely that all the GluCl subunits are known (The *C. elegans* Sequencing Consortium, 1998). However in *D. immitis* the only subunits that have been identified are GluClα3A and α3B. As initially stated, it is possible that these subunits may associate with each other to form heteromers, a hypothesis that can be readily tested. It may also be possible to express these subunits with GluCl subunits from other species. If seemingly genuine associations of the CeGluClα3 subunits with other *C. elegans* GluCl polypeptides were made, similar experiments could be conducted substituting the *D. immitis* subunits for their orthologues. As discussed in section 4.3, the value of these experiments is debatable given there is no evidence to suggest that the other *C. elegans* GluCl subunits have orthologues in *D. immitis* let alone evidence regarding matters of co-localisation. Certainly, it seems the GluCl subunit genetics between even closely related species of nematode are not highly conserved; the HcGluClα subunit has no direct orthologues in *C. elegans*. In addition, expression patterns of the orthologous GluClα3 subunits between *C. elegans* and *H. contortus*, whilst consistent in parts also exhibit considerable differences (Dent *et al.*, 2000; Portillo *et al.*, 2003).

The above discussion regarding future work will be academic unless the expression of the *C. elegans* and especially the *D. immitis* GluClα3 subunits in *Xenopus* oocytes can be

improved. Clearly the addition of the 5'-AMV sequence improved expression of the polypeptides, as during the initial trial experiments no glutamate-sensitive oocytes were observed, even with those injected with CeGluClα3B cRNA. However, the expression obtained following the inclusion of this sequence was still inconsistent; oocytes injected with CeGluClα3 cRNA from batch to batch varied considerably in their response to glutamate while few oocytes injected with DiGluClα3B cRNA responded to this ligand at all. It is quite probable that some of the expression inconsistencies were due to a natural variation in oocyte quality from one frog to the next. However, there remain a number of steps that could be taken, at the DNA/RNA level to improve expression. As illustrated by the 5' AMV sequence, expression of foreign RNA in oocytes can be heavily influenced by 5' and 3' untranslated sequences. Therefore, one way of increasing expression maybe to sub-clone the subunit cDNAs into specialist *Xenopus* oocyte expression vectors, such as pSP64T (or one of its derivatives). Coding sequences inserted into these vectors are flanked by 5' and 3'-untranslated regions derived from the *Xenopus* β-globin mRNA, which appear to significantly enhance the foreign RNA's translation efficiency in this system (Krieg & Melton, 1984). Vectors of this type have been used in the expression of the CeGluClα2A and HcGluClα subunits in oocytes (Dent *et al.*, 1997; Forrester *et al.*, 2003). An alternative method, which was attempted to increase expression of the DiGuClα3 subunits in mammalian cell-lines, would be to incorporate a Kozak sequence directly upstream of the start codon; Kozak sequences promote efficient translation from whichever ATG codon they are located next to (Kozak, 1986). Polyadenylation of cRNA is another way shown to improve stability and translational efficiency. This can be achieved by incorporating such a region into the vector at the 3' end of the multiple cloning site (pSP64T has one), hence it is added during transcription, or can be added post-transcription using an RNA poly(A) polymerase. Whilst incorporation of this sequence has improved expression for some proteins it certainly does not stabilise or promote translation of all foreign mRNAs injected into oocytes (Wormington, 1991). An alternative method to cRNA injected into the cytoplasm, is to inject the subunit cDNA into the nucleus of the oocyte. Although more technically difficult, by doing this one bypasses the need for the *in vitro* procedures required for preparing cRNA and the inherent problems associated with possible degradation (Betrand *et al.*, 1991). As discussed in section 4.3, it might be interesting whether codon optimisation for the GluClα3 subunit cDNAs may improve expression in

Xenopus oocytes as has been shown for *C. elegans* GluCl subunit cDNAs in mammalian cells (Slimko & Lester, 2003). This may be particularly important given the striking AT bias in *D. immitis* codons (Fadiel *et al.*, 2001).

In summary, the responses (or lack of) elicited by glutamate and IVM applied to oocytes injected with either DiGluClα3A or α3B cRNA were consistent with the known pharmacology of orthologous subunits. DiGluClα3B formed homomeric channels gated by both glutamate and IVM whilst in DiGluClα3A cRNA injected oocytes no currents were observed upon application of glutamate at high concentrations; the IVM response in these cells remains to be tested. As discussed above it is unknown whether the DiGluClα3A subunits are even able to associate into homomers. The GluClα3B subunit is therefore a potential molecular target for the AMs in *D. immitis*. But how sensitive is DiGluClα3B to AMs? And where and at which developmental stage(s) is the subunit expressed? Questions like this need to be addressed to establish how important (or not) this subunit is in conveying the sensitivity of the larval stages of *D. immitis* to the AMs. Additionally, answers to these questions may indicate why these drugs are so ineffective at killing adult worms. Studies using oocytes have also allowed a glutamate dose response curve to be determined for CeGluClα3B homomers, the EC_{50} and Hill coefficients generated being comparable to other GluCls in oocytes. However they also indicate that this subunit probably does not form homomers *in vivo*. This is probably true for the CeGluClα3A subunits also. Unfortunately, not all of the original objectives outlined in the introduction were achieved, which was mainly due to the highly variable expression of the subunits in the oocytes. Hopefully, the measures suggested to overcome these problems, will aid enable further pharmacology of these subunits to be unraveled.

Chapter 6

Final Discussion

6.1 DiGluClα3B is a molecular target for the AMs

D. immitis is a filarial nematode that primarily infects domesticated dogs causing cardiopulmonary disease. In severe cases this can lead to right-sided heart failure. It is a widely distributed parasite and hence drugs used to combat infections are of significant economic importance in the rapidly expanding animal health care market (Witty, 1999). Chemoprophylaxis is the most effective and safest way of controlling heartworm disease. The major drugs used in this preventative treatment are the AMs. Although these compounds are extremely effective at killing larval forms of the parasite, they are ineffective at killing adult worms, hence their roles as prophylactics (Knight, 1998). The overall objective of this study was to address why this may be so by establishing which molecules are the likely targets for such drugs. It is hoped that a greater understanding of the target molecules might, in the future, direct the development of improved therapies that are also effective against the adult worms. This is important, as the treatments available to remove the adult worms are also very toxic to the host (Knight, 1998). Numerous studies have indicated that the GluCls are the targets for the AMs (see section 1.8.3). Therefore the aims of the study were to firstly clone GluCl subunit cDNAs from *D. immitis* and then express the polypeptides in order to investigate their pharmacology with respect to the AMs. The results of this study, summarised below, indicate that these aims have largely been achieved.

From the research undertaken two GluCl subunit cDNAs were cloned. Based on homology searches, the encoded subunits are clearly orthologous to the alternatively spliced GluClα3 subunits (from the *avr-14* gene) identified in *C. elegans* and *H. contortus* (Laughton *et al.*, 1997a; Jagannathan *et al.*, 1999). The *D. immitis* GluClα3 subunits failed to express at detectable levels in mammalian cell-lines. Therefore TEVC recordings were made from *Xenopus* oocytes injected with subunit-specific cRNA to investigate their pharmacology. The GluClα3B subunits formed channels that were gated by glutamate (1-100mM) and IVM (1μM), in typical fashions, establishing this molecule as a potential target for the AMs *in vivo*. However, oocytes injected with GluClα3A cRNA failed to respond to glutamate; the response to IVM in these oocytes was not tested. The qualitative responses obtained were consistent with the pharmacology observed for the GluClα3 subunits in *C. elegans*

(Dent *et al.*, 2000; this study) and *H. contortus* (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002), perhaps not surprising given the high degree of amino acid identity (≥80%) between orthologues.

The next stage of achieving the original objective would be to ascertain if the GluClα3B subunit is an important target for AMs. As outlined in the last chapter, this would entail addressing a number of questions including how sensitive the receptors formed from these subunits are to the AMs? When during the lifecycle, i.e. during which developmental stages, are the subunits expressed? Where in the worms are they expressed? And finally, does their tissue distribution correlate well with the typical biological activities displayed by the AMs, i.e. inhibition of pharyngeal pumping, motility and fecundity? Without addressing such questions there remains a number of explanations for why these compounds exhibit such different potencies against larvae and adult worms. An obvious and rather simple explanation could be that while larval worms express AM-sensitive GluCls, adult worms do not; the CeGluClβ subunit has been shown to form functional channels that are glutamate but not IVM sensitive (Cully *et al.*, 1994). The GluClα3 subunit cDNAs were amplified from mRNA isolated from adult female *D. immitis*. However, the presence of unreleased microfilaria within these worms makes it impossible to draw many conclusions as to the stage specific expression of these subunits. An alternative explanation could be that all stages express AM-sensitive GluCls, but in adults their expression pattern may not correlate well with the processes typically affected by the AMs in other species (and presumably *D. immitis* larvae), i.e. activation of GluCls does not lead to an inhibition of pharyngeal pumping or motility. Indeed, the expression pattern of the GluClα3B subunits appears not to be absolutely conserved between even closely related species (Dent *et al.*, 2000; Portillo *et al.*, 2003). Of course it remains possible that inhibition of these processes in adult *D. immitis* is simply not important; the worms do not have to constantly move to fight against forces such as the peristaltic flow in the gut and appear to be able to derive their nutrients through transcuticular absorption rather than via the pharynx (Geary *et al.*, 1995). One final explanation could be that the drugs are simply unable to access their target, although transcuticular absorption of IVM uptake in other species of nematode has been demonstrated (Smith & Campbell, 1996).

6.2 What is the true molecular mechanism of AM action?

Aside from establishing whether the GluClα3B subunit is potentially an important target for the AMs it would be interesting to further investigate the true mechanism of action of these compounds in respect to the GluCls. As stated, there is a clear discrepancy between the high concentration of AMs required to activate recombinant GluCls expressed in oocytes (>10nM) (Arena *et al.*, 1992; Cully *et al.*, 1994; Cully *et al.*, 1996a; Dent *et al.*, 1997; Vassilatis *et al.*, 1997a; Horoszok *et al.*, 2001; Forrester *et al.*, 2003) with the significantly lower concentrations able to bind individual GluCl subunits (pM range) (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002) and those that exert activity towards worms *in vitro* (≥0.1nM) (Kass *et al.*, 1980; Avery & Horvitz, 1990; Gill *et al.*, 1991, 1995; Geary *et al.*, 1993; Arena *et al.*, 1995; Paiement *et al.*, 1999). Low concentrations of IVM (<10nM) have been reported however, to potentiate sub-maximal concentrations of glutamate in oocytes injected with selected GluCls (Cully *et al.*, 1994, 1996b; Forrester *et al.*, 2003).

A recent electrophysiological study demonstrated that the native pharyngeal GluCls from *C. elegans* were considerably more sensitive to IVM than GluCls expressed in *Xenopus* oocytes (Pemberton *et al.*, 2001). The potency of the IVM response (EC_{50}=2.7nM) was comparable with concentrations shown to paralyse the pharynx *in vitro* (Avery & Horvitz, 1990; Geary *et al.*, 1993) and bind individual GluCl subunits (Cheeseman *et al.*, 2001; Forrester *et al.*, 2002). Two explanations could account for this result, as outlined in Pemberton *et al* (Pemberton *et al.*, 2001). Firstly, the differing IVM sensitivities could be explained by the subunit composition of the native receptors. Only a limited number of subunit combinations have been successfully expressed so far in oocytes and none of these may reflect the stoichiometry of native the GluCl. Secondly, and perhaps more intriguingly, the potency observed reflected the potentiation of the IVM response by endogenous glutamate. In support of this, a recent study has shown that glutamate potentiated AM binding to the *H. contortus* HcGluClα subunit (Forrester *et al.*, 2002).

Therefore, the pharmacological evidence suggests that while GluCls are the high-affinity molecular targets for the AMs in nematodes, glutamate does not share the same binding

site; glutamate does not compete for IVM binding-sites in membrane preparations (Schaeffer & Haines, 1989). This is perhaps not surprising given the highly lipophilic nature of the AMs (Burkhart, 2000). The potentiation of the electrophysiological response to glutamate by IVM and of IVM binding by glutamate, indicate an allosteric relationship between the glutamate and ivermectin binding sites. How these potentiation effects relate to the AMs ability to directly activate the channels is not easily reconciled. It is tempting to speculate that the direct activation of the channels is due to a second, lower affinity, AM binding site on the receptor. A site with an affinity close to the observed EC_{50} for IVM on oocyte-expressed GluCl (~100nM) would not have been detected in the binding experiments of Cheeseman *et al* (2001) or Forrester *et al* (2002), in which the highest concentrations of radioligand used were <5nM. Whether it is a direct activation of GluCl or potentiation of glutamate responses, or both, that is important for AM anthelmintic activity *in vivo* is unknown.

6.3 Summary of other results

Complimentary TEVC studies were conducted on the *C. elegans* GluClα3B subunits, expressed as homomers in oocytes. The glutamate dose response curve generated for these channels had an EC_{50} value of 2.2 ± 0.12mM and a Hill coefficient of 0.72 ± 0.08. As discussed in section 5.3, the EC_{50} value is comparable to those determined for GluClα2A and GluClα4 homomers (Dent *et al.*, 1997; Horoszok *et al.*, 2001). However, it is significantly higher than EC_{50} values determined for oocytes injected with *C. elegans* mRNA (Arena *et al.*, 1992) and the GluCl described in the *C. elegans* pharynx (Pemberton *et al.*, 2001). This suggests CeGluClα3B subunits do not form homomeric receptors *in vivo*. Interestingly, the Hill coefficient for CeGluClα3B homomers indicated that only one glutamate molecule was able to activate the receptor. This is unusual compared with other GluCls (see table 1.2) and may reflect an underestimation of the true value caused by the rapid desensitization of the receptors in the presence of glutamate.

During the original cloning phase of the project, numerous PCRs were conducted using degenerate oligonucleotide primers in an attempt to amplify further *D. immitis* GluCl cDNAs. Although no new GluCl cDNAs were amplified, a potentially novel LGIC subunit cDNA, termed DG1, was isolated. The subunit exhibited low amino acid identity with other

LGCCs (20-30%), but exhibited ~50% identity to a predicted LGIC subunit in *C. elegans* (T27A1.4). DG1, and indeed T27A1.4 may represent outlying members of the LGIC superfamily; the contrasting conserved nature of certain characteristic features of these channels (i.e. second "cys-loop" and apparent 4 TM regions) against the absence of such a defining feature as the first "cys-loop" remains a conundrum. Interestingly, there appears to be a related sequence in the *Brugia malayi* genome.

6.4 Final conclusions

During this study, two *D. immitis* GluCl subunit cDNAs have been amplified, the encoded subunits being clearly orthologous to the alternatively spliced GluClα3 subunits from *C. elegans* and *H. contortus*. The *D. immitis* GluClα3B subunit has been identified as a potential molecular target for the AMs *in vivo*, by forming IVM-sensitive homomeric channels in oocytes. As discussed, it will be interesting to examine if this subunit plays an important role in explaining why the AMs exhibit such different potencies against adult and larval forms of *D. immitis*. From a commercial aspect, this subunit may now be used as a molecular target in screens to develop new or existing anthelmintic compounds.

Interest in GluCls as molecular targets partially stems from the fact that they are only found in invertebrates, a particularly advantageous characteristic with regards to the design of anthelmintic drugs. It follows, that if the drugs target molecules only found in the parasite then there is decreased likelihood of side effects (caused by the drug) in the host. Therefore it will be very interesting to see if the DG1 or T27A1.4 subunits are important for the viability of their respective species of nematode. They both appear to be highly unusual molecules and thus would be ideal as potential targets for the development of novel anthelmintics.

Appendices

Appendix A
Oligonucleotide primer details

From chapter 3:

Primers used to amplify DiGluClα3 subunit cDNAs

Primer	Nucleotide sequence (5'→3')	Comments
5Di2/3P1	gaagcaagaagacatcttattgacaag	Internal sense primer
3Di2/3P1	gctatattcacctgtatttgttttactcg	Internal anti-sense primer
SL1	ggtttaattacccaagtttgag	SL1 sense primer
RoRi	gactacgttagcatctagaattctcgag	RiRo adaptor site anti-sense primer
SDG2	ctattatgaatcctcgtatagtttattgg	α3A/B coding region sense primer
ASDG2	ccaagcttttgttaaatgaggtaaacag	α3A coding region anti-sense primer
ASDi2/3P2	gtgcactatattccatgttaacgtcatc	Internal anti-sense sequencing primer
ASDG3	cttcatgttaattgacataattcac	α3B coding region anti-sense primer

Degenerate primers

Primer	Region designed to	Nucleotide sequence (5'→3')
SDGAP1	YIKAIDVW	tatat(atc)aaagc(at)at(atc)ga(tc)gtntgg
SDGAP2	HV/IPIEQPQT	cat(ga)t(ta)cc(ta)at(tca)gaaca(ag)cc(at)ca(ag)ac
SDGAP3	PMH/YLQYYPMD	cc(at)atg(tc)at(tc)tnca(ga)tattatccnatgg
ASDGAP1	WKWIKTKTE	c(gc)gtttt(gc)gtttt(agt)atcca(tc)ttcc
ASDGAP2	WMPDTFFP/Q	tg(ag)aa(ag)aa(ta)gtatc(cta)ggcatcc
ASDGAP3	PMH/YLQYYPMD	ccat(at)ggataata(ct)tgna(ga)(ga)t(ga)catngg
SDGBP1	YVKVVDVW	tatgtnaaagtngtnga(tc)gtntgg
ASDGBP1	PDYLPAKI	atttt(at)gc(cta)ggna(ag)(ag)ta(ag)tcngg
ASDGBP2	WIPDTFFP	gg(ag)aa(ag)aa(ta)gtatc(cta)gg(agt)atcc

The primers in blue font represent those designed with bias towards the GluClα-type subunits. The primers in red font represent those designed with bias to the GluClβ-type subunits. "S" prefix denotes the sense primers whilst "AS" prefix denotes the anti-sense primers. The regions the primers were designed to are indicated in appendix C.

Primers used to amplify DG1 and T27A1.4 subunit cDNAs

Primer	Nucleotide sequence (5'→3')	Comments
SL1	ggtttaattacccaagtttgag	SL1 sense primer
RoRi	gactacgttagcatctagaattctcgag	RiRo adaptor site anti-sense primer
SBETA2P2	ggtctcgagggtcaggtgctg	Internal sense primer
SDG1	gcaatgtggtcaacaattgtatcg	Coding region sense primer
ASDG1	gacgaatgtatcagtcaattatac	Coding region anti-sense primer
SBETA2P3	cggttgttgaaccaaccggatatg	Internal sequencing primer
ST27A1.4	atgaggaacattttcttatttctagtacta	Coding region sense primer
AST27A1.4	ttaaacaataaaaataaagtaaataatcaa	Coding region anti-sense primer
T27seq1	gtcagcaatggcaggatagtcgtttg	Internal sequencing primer
T27seq2	gagaagaaggtgagcacaaggcag	Internal sequencing primer

From chapter 4:

Primers used to amplify DiGluClα3 cDNAs for cloning into the epitope-tag vectors

Primer	Nucleotide sequence (5'→3')
SDG2TAG	nnnnnn*ctgcag*atgaatcctcgtatagtt (PstI)
ASDG2TAG	nnnnnn*ggatcc*aatgaggtaaacagc (BamHI)
SDG2TAGOP	nnnnnn*ctgcag***gccacc**atgaatcctcgtatagtt (PstI)
ASDG3TAG	nnnnnn*ctcgag*attgacataattcacataataaag (XhoI)

Underlined italic sequence indicates the position of the incorporated restrictions site given in brackets. The Kozak sequence is highlighted in bold type.

From chapter 5:

Primers used to amplify the AMV-GluClα3 subunit cDNAs

Primer	Nucleotide sequence (5'→3')
SDiGluClα3AMV	nnnnnn*tctagag***ttttttatttttaattttctttcaaatacttccat**atgaatcctcgtatagttc (XbaI)
ASDiGluClα3AAMV	nnnnnn*gcggccgc*ttaaatgaggtaaacagccca (NotI)
ASDiGluClα3BAMV	nnnnnn*gcggccgc*ttaattgacataattcacata (NotI)
SCeGluClα3AMV	nnnnnn*tctagag***ttttttatttttaattttctttcaaatacttccatc**atgtggcattatcgactg (XbaI)
ASCeGluClα3AAMV	nnnnnn*gcggccgc*tcacatcaggtagacggc (NotI)
ASCeGluClα3BAMV	nnnnnn*gcggccgc*ttaattgacataattcacata (NotI)

Underlined italic sequence indicates the position of the incorporated restrictions site given in brackets. The AMV sequence is highlighted in bold type.

Appendix B
Nucleotide sequences of cloned *D. immitis* LGIC subunit cDNAs

The black sequence indicates the coding-region. The blue region is the SL1 sequence and the red regions the untranslated sequence. The proposed start codon is highlighted in yellow.

>GluClα3A

```
   1 GGTTTAATTA CCCAAGTTTG AGGAAAATTT ACTATTATGA ATCCTCGTAT AGTTTATTGG
  61 AATCTCATTC TTCTAATCCT TGTTGTGGCA AAGAAAAAAC TGAAAGAGCA AGAAATAATC
 121 CAGAGAACGT TGAAGGATTA CGATTGGCGT GTACGACCAC GTGGCAGTAA TCTTTCCTGG
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1441 TTATACCGAA TATATCTATA CATATATTCC TTCTTCATTT TCTA
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>GluClα3B

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Appendix C
Alignment of GluCl subunit amino acid sequences

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DiGluClα3A      ..................................................
DiGluClα3B      ..................................................
CeGluClα3A      ..................................................
HcGluClα3A      ..................................................
CeGluClα3B      ..................................................
HcGluClα3B      ..................................................
AsGluClX        ..................................................
OvGluClX        ..................................................
CeGluClα1       ......................MATWIVGKLIIASLILGIQAQQARTKSQ      28
CeGluClα2B      ......................MSTSFIRRLAFVGLLLGVHAYHSRPKSE      28
CeGluClα4       ..................................................
HcGluClα        ..................................................
CeGluClβ        ..................................................
HcGluClβ        ..................................................
DmGluClα1       ..................................................
hGlyRα1         ..................................................
rGABA_Aα1       ..................................................
CeGLC4          MFSSVFNFQQFLFGILIRLCLFVDLDDLHNPSTDHLPLVPSRRESARNAK      50
mGABA_Aβ2       ..................................................

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DiGluClα3B      ....................MNPRIVYWNLILLIL..VVAKKKLKEQEII      28
CeGluClα3A      ....................MWHYRLTTILLIISIIHSIRAKRKLKEQEII      31
HcGluClα3A      ..........MRNSVPLATRIGPMLALICTVSTIMSAVEAKRKLKEQEII      40
CeGluClα3B      ....................MWHYRLTTILLIISIIHSIRAKRKLKEQEII      31
HcGluClα3B      ..........MRNSVPLATRIGPMLALICTVSTIMSAVEAKRKLKEQEII      40
AsGluClX        ..................................................
OvGluClX        ....................MNSFPIVCWNLAFLIL..VVAKKKLKEQEII      29
CeGluClα1       ...........DIFEDDNDNGTTTLESLARLTSPIHIPIEQPQTSDSKIL      67
CeGluClα2B      EHSPKHAASSSDLFEDDDQS..TTLESIARLSAPAHVPIEQPQTSDTEIL      76
CeGluClα4       ....................MSLRSLLNILLIVAFWIVGGNCDASSDTEII      31
HcGluClα        ....................MFALILPFLLHFTRSEGFGYEKLLDEQKII      30
CeGluClβ        ............MTTPSSFSILLLLLLMPVVTNGEYSMQS.....EQEIL      33
HcGluClβ        ................MSQYMMVAVAAVVAVAGSSQISRRSTGGTQEQEIL      35
DmGluClα1       ............MGSGHYFWAILYFASLCSASLANNAKVNFREKEKK.VL      37
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rGABA_Aα1       ........MKKSRGLSDYLWAWTLILSTLSGRSYGQPSQDELKDNTTVFT      42
CeGLC4          ETVYHRLFFVMKAQLYVSVLLALLVSSTAKKSKTKSCKRTAFSRHTTNYQ     100
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CeGluClα3B      Q....RILK....DYDWRVRPRGMNATWPDTG...GPVLVTVNIYLRSIS      70
HcGluClα3B      Q....RILN....NYDWRVRPRGLNASWPDTG...GPVLVTVNIYLRSIS      79
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OvGluClX        Q....RTLK....DYDWRVRPRGNNLSWPDTG...GPVLVSVNIYLRSIS      68
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CeGluClα2B      E....HLLTR...GYDHRVRPPGEDGTIHG.....GPVVVSVNMLLRSIS     114
CeGluClα4       K....KLLGK...GYDWRVRPPGINLTIPGTH...GAVIVYVNMLIRSIS      71
HcGluClα        K....HLLESPYSDYDWRVRPRGRLGPADDDDYDSEPVFITVNMYLRSIS      76
CeGluClβ        N....ALLK....NYDMRVRPPPAN.SSTE.....GAVNVRVNIMIRMLS      69
HcGluClβ        N....ELLS....NYDMRVRPPPSNYSDPM.....GPVTVRVNIMIRMLS      72
DmGluClα1       D.....QILG.AGKYDARIRPSGING...TD....GPAIVRINLFVRSIM      74
hGlyRα1         D.....KLMGRTSGYDARIRP...NF...KG....PPVNVSCNIFINSFG      77
rGABA_Aα1       R.....ILDRLLDGYDNRLRP...GL...GE....RVTEVKTDIFVTSFG      77
CeGLC4          AWREQMTVCDLLQDYDAAVRPSGRTPYNDTR....GAVMVTTSLNIRSIS     146
mGABA_Aβ2       ......TVDRLLKGYDIRLRPDFGGP..........PVAVGMNIDIASID      72
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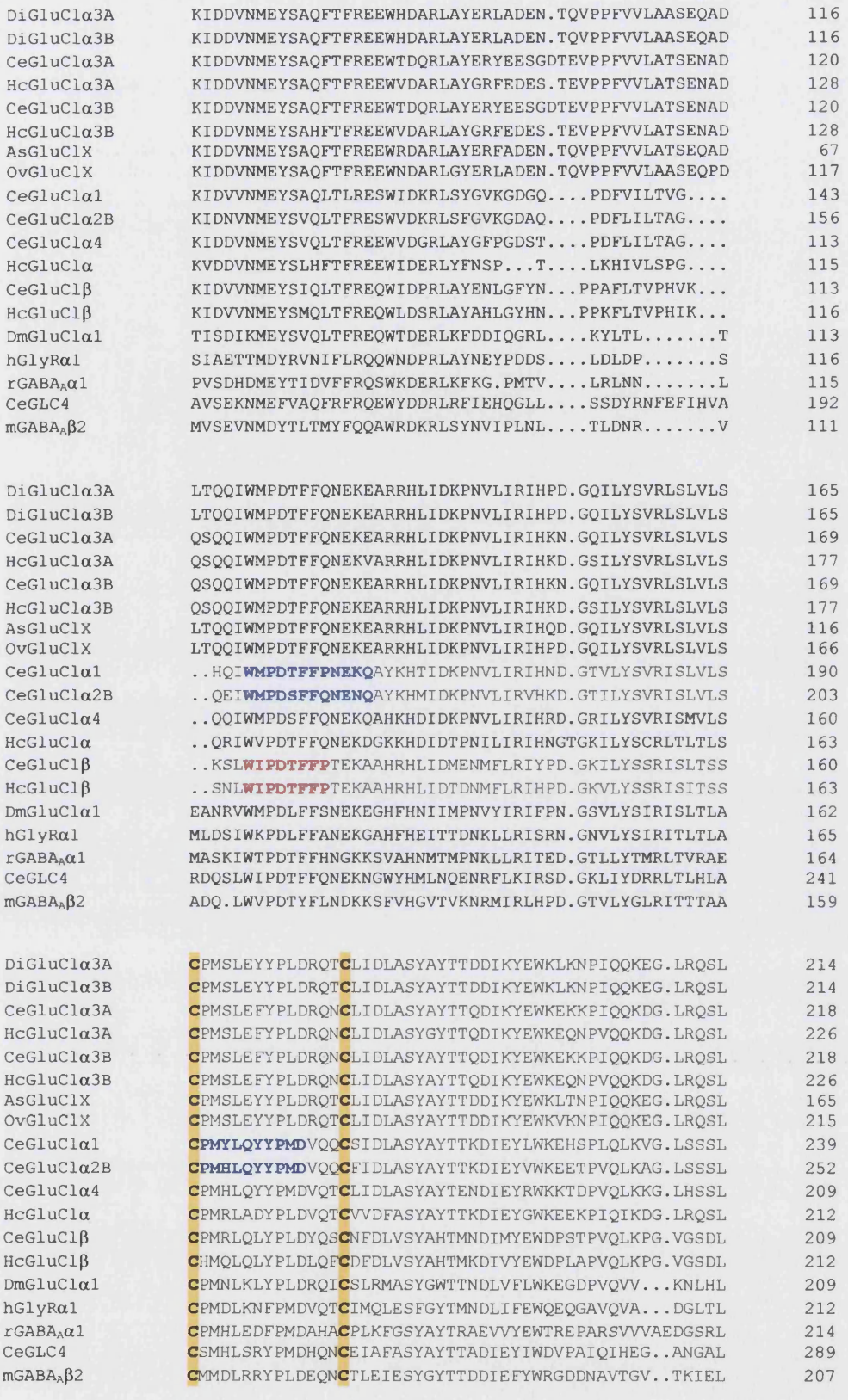
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HcGluClα3A KIDDVNMEYSAQFTFREEWVDARLAYGRFEDES.TEVPPFVVLATSENAD 128
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CeGluClα4 ..QQIWMPDSFFQNEKQAHKHDIDKPNVLIRIHRD.GRILYSVRISMVLS 160
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TM2

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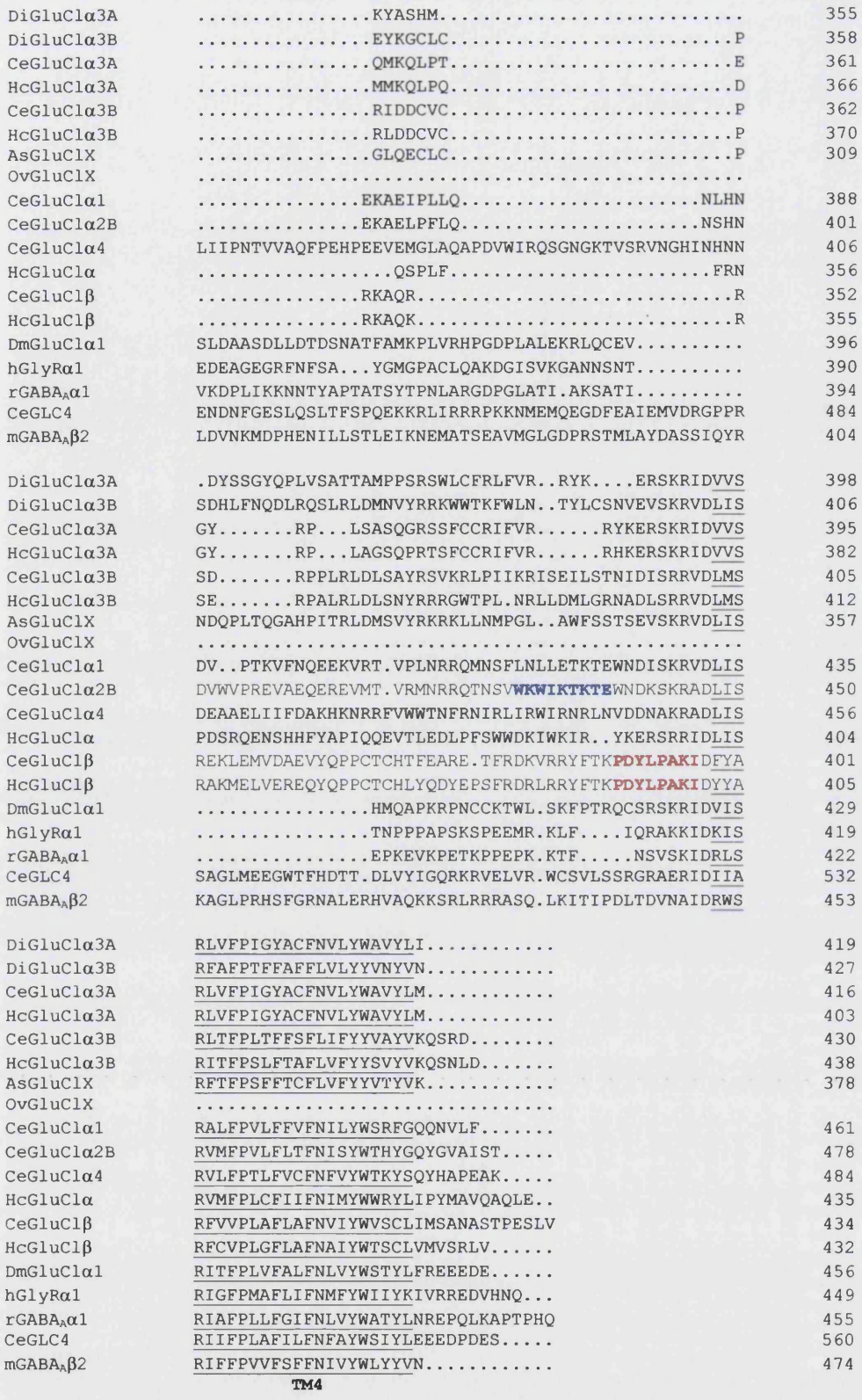
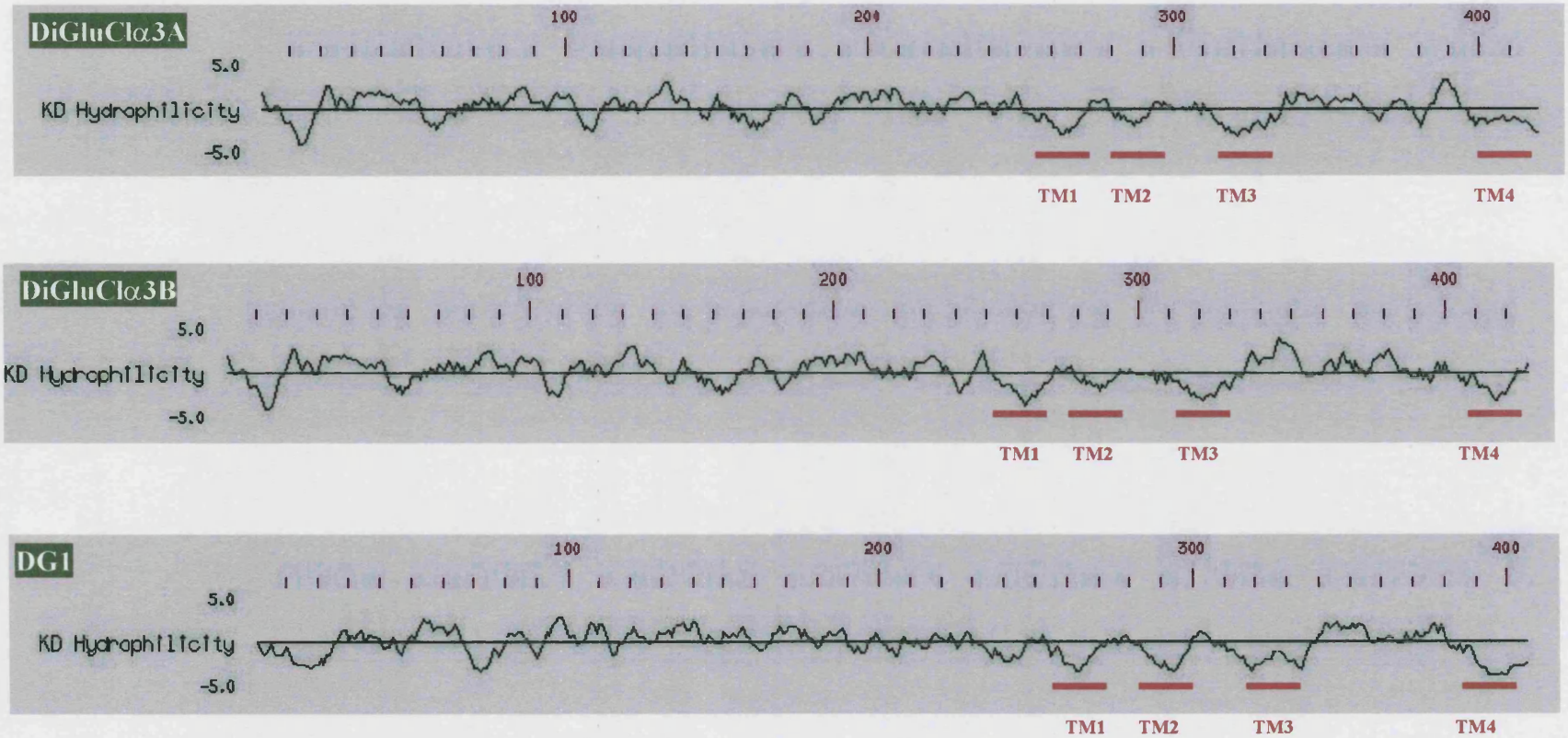
CeGluClβ VDVWLGACQTFVFGALLEYAFVSYQDSVRQNDRSREKAA..... 346
HcGluClβ VDVWLGACQTFVFGALLEYAFVSYQDSQRQTEQAKSRAA..... 349
DmGluClα1 IDVWTGVCLTFVFGALLEFALVNYASRSGSNKANMHKENMKKKRRDLEQA 356
hGlyRα1 IDIWMAVCLLFVFSALLEYAAVNFVSRQ.....HKELLRFRRKRRHHK 353
rGABA$_A$α1 MDWFIAVCYAFVFSALIEFATVNYFTKRG.....YAWDGKSVVPEKPKK 355
CeGLC4 IDIWVGACVVFIFFSLIEYAVVNYVGILDEHRQMKKAACNRSRLSN..VI 434
mGABA$_A$β2 IDMYLMGCFVFVFMALLEYALVNYIFFGRGPQRQKKAAEKAANANNEKMR 354
TM3



Cysteine residues that form the disulphide bridges are highlighted in yellow (indicative of the LGICs) and green (indicative of the GluCls and glycine receptors) while the underlined regions indicate the TM regions. The residues in red and blue font are the regions to which the degenerate primers were designed (see appendix A).

Appendix D
Hydropathy plots for the DiGluClα3 and DG1 subunits



The hydropathy plots were constructed using the GCG program "PeptideStructure"; this program uses the Kyte-Doolittle method for calculating hydrophilicity (Kyte & Doolittle, 1982). The numbered bar indicates the amino acid residue. The jagged line deviating from the horizontal black bar is an indication of the hydropathy of the amino acid sequence. Upward and downward deflections from the bar represent hydrophilic and hydrophobic residues respectively. The proposed hydrophobic TM regions are marked on the plot with numbered horizontal red bars.

Appendix E
D. immitis codon frequency table

Amino acid	Codon	%		Amino acid	Codon	%
Alanine	GCG	15		Leucine	CTA	9
Alanine	GCA	42		Leucine	CTT	17
Alanine	GCT	32		Leucine	CTC	9
Alanine	GCC	11				
				Lysine	AAG	27
Arginine	CGG	13		Lysine	AAA	73
Arginine	CGA	16				
Arginine	CGT	20		Methionine	ATG	100
Arginine	CGC	5				
Arginine	AGG	19		Phenylalanine	TTT	67
Arginine	AGA	28		Phenylalanine	TTC	33
Asparagine	AAT	73		Proline	CCG	20
Asparagine	AAC	27		Proline	CCA	44
				Proline	CCT	25
Aspartate	GAT	80		Proline	CCC	11
Aspartate	GAC	20				
				Serine	AGT	16
Cysteine	TGT	53		Serine	AGC	16
Cysteine	TGC	47		Serine	TCG	15
				Serine	TCA	29
Glutamate	GAG	15		Serine	TCT	12
Glutamate	GAA	85		Serine	TCC	11
Glutamine	CAG	26		Threonine	ACG	19
Glutamine	CAA	74		Threonine	ACA	36
				Threonine	ACT	25
Glycine	GGG	10		Threonine	ACC	19
Glycine	GGA	36				
Glycine	GGT	33		Tryptophan	TGG	100
Glycine	GGC	21				
				Tyrosine	TAT	71
Histidine	CAT	74		Tyrosine	TAC	29
Histidine	CAC	26				
				Valine	GTG	18
Isoleucine	ATA	22		Valine	GTA	24
Isoleucine	ATT	54		Valine	GTT	41
Isoleucine	ATC	24		Valine	GTC	18
Leucine	TTG	30		Stop	TGA	58
Leucine	TTA	21		Stop	TAG	9
Leucine	CTG	13		Stop	TAA	33

The codon frequency table was constructing using 15 *D. immitis* mRNA sequences and the GCG program "CodonFrequency". The GenBank accession numbers of the nucleotide sequences used are: Af000668, Af001007, Af004167, Af008300, Af019884, Af027387, U04693, U14753, U29459, D88757, M82811, U29533, U447813, U87457.

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